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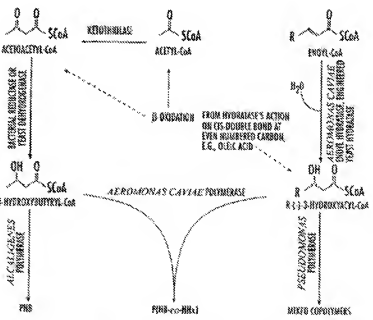

(57) Abstract: The invention relates to the genetic manipulation of plants to produce polyhydroxyalkanoate, particularly in the peroxisomes. Methods for producing such polymers in plants and host cells are provided. Such methods find use in producing biodegradable thermoplastics in plants and other organisms. Nucleotide molecules, expression cassettes, and genetically manipulated host cells, plants, plant tissues, and seeds are also provided.

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PRODUCTION OF POLYHYDROXYALKANOATE IN PLANTS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Patent Application No. 60/156,807, filed September 29, 1999.

FIELD OF THE INVENTION

The present invention relates to the genetic manipulation of plants for the production of biodegradable thermoplastics, particularly polyhydroxyalkanoate copolymers.

BACKGROUND OF THE INVENTION

Composed of polymers of a variety of organic compounds, plastics can be molded, extruded, cast into various shapes and films, and even drawn into fibers. It is such versatility that has led to incorporation of plastics into a seemingly endless number of products. Thus, plastic products have become an integral part of everyday life in industrialized society, and the demand for these products is expected to grow as the world population grows and developing countries move up the economic ladder. However, synthetic plastics are slow to degrade in landfills. If and when they do breakdown, the monomers and their derivatives resulting from degradation may actually be more hazardous to human health than the undegraded polymers (Selenskas *et al.* (1995) *Amer. J. Indust. Med. 28*:383R-398; Tosti *et al.* (1993) *Toxicol. Indust. Health 9*:493-502; Yin *et al.* (1996) *J. Food Drug Anal. 4*:313-318). These concerns have raised to a new level the urgency of exploring the use of the environmentally friendly, compostable polymers as substitutes for synthetic plastics.

Polyhydroxyalkanoates (PHAs) are polyesters of hydroxyalkanoic acids that are synthesized by a variety of bacteria as storage polymers under stressful conditions (Steinbuchel, A. (1991) *Biomaterials: Novel materials from biological materials*, D. Byrom, ed. (New York: Macmillan Publishers Ltd.), pp. 123-213). Since PHAs have thermoplastic properties, that is they become soft when heated and hard when cooled, and are fully biodegradable, they offer an attractive alternative to synthetic plastics (Brandl *et al.* (1995) *Can. J. Microbiol. 41*: 143-153; Byrom, D. (1993) *Int. Biodeterior. Biodegrad. 31*:199-208; Lee, S.Y. (1996) *Biotechnol. Bioeng. 49*:1-14;

Nawrath *et al.* (1993) *Abst. Pap. Amer. Chem. Soc. 206*:22-27; Poirier *et al.* (1995) *Bio/technology Nat. Publ. Co. 13*:142-150; Steinbuechel, A. (1992) *Curr. Opin. Biotechnol. 3*:291-297). Unlike man-made plastics, the production of PHA by living organisms is not dependent on finite natural resources like petroleum.

Currently, only one type of polyhydroxyalkanoate (PHA), Biopol, a copolymer made by fermentation, is commercially available (Poirier *et al.* (1995) *Bio/technology Nat. Publ. Co. 13*:142-150). However, at approximately $7 per pound, this polymer is much too expensive in comparison to the synthetic plastics that have similar properties but are cheaper with a price of approximately $0.5 per pound (Poirier *et al.* (1995) *Bio/technology Nat. Publ. Co. 13*:142-150). The higher cost of Biopol results primarily from its cost of production, the main contributing factor being the substrate (Poirier *et al.* (1995) *Bio/technology Nat. Publ. Co. 13*:142-150). If the PHAs can be produced in plants, the cost of production can be lowered substantially because these polymers would compete with seed oil as natural storage constituents of the cell. The current market price of plant seed oil is between 26 and 28 cents per pound (Anonymous (1998) *Economic Research Service* (Washington, DC 20036: U.S. Department of Agriculture). Only about 40% of the energy required to extend a fatty acid chain by two carbons is expended on extending a PHA chain by the same length. Starting with acetyl-CoA, a two carbon extension in oil biosynthesis requires two NADPH and one ATP. In comparison, only one NADPH is needed to accomplish the same for PHA biosynthesis (Figure 1). Theoretically, more than two units of PHA should be formed for every unit of oil replaced.

Until recently, the only PHA that has been produced in plants was polyhydroxybutyrate (PHB), a homopolymer of 3-hydroxybutyric acid (John *et al.* (1996) *Proc. Natl. Acad. Sci. USA 93*:12768-12773; Nawrath *et al.* (1994) *Proc. Natl. Acad. Sci. USA 91*:12760-12764; Padgette *et al.* (1997) *Plant Physiol. 114* (Suppl.) 3S; Poirier *et al.* (1992) *Science 256*:520-523)). Because this polymer is crystalline and brittle with a melting point too close to its degradation point, PHB is difficult to mold into desirable products (Lee, S.Y. (1996) *Biotechnol. Bioeng. 491:1-14)*. Many bacteria make copolymers of 3-hydroxyalkanoic acids with a carbon chain length greater than or equal to five (Steinbuchel, A. (1991) *Biomaterials: Novel materials from biological materials*, D. Byrom, ed. (New York: Macmillan Publishers Ltd.), pp. 123-213). Such copolymers are polyesters composed of different 3-hydroxyalkanoic

acid monomers. Depending on the composition, these copolymers can have properties ranging from firm to elastic (Anderson *et al.* (1990) *Microbiol. Rev. 54*:450-472; Lee, S.Y. (1996) *Biotechnol. Bioeng. 49*:1-14). Unlike PHB, the PHA copolymers are suitable for a variety of applications because they exhibit a wide range of physical properties.

Initial attempts at producing PHA in the cytosol proved toxic to the plant (Poirier *et al.* (1992) *Science 256*:520-523). This problem was overcome by targeting the PHA-producing enzymes to plastids (Nawrath *et al.* (1994) *Proc. Natl. Acad. Sci. USA 91*:12760-12764). In either cellular compartment, however, only PHB was accumulated, not any of the copolymers. With both of these methods, the genes from *Ralstonia eutropha* (also known as *Alcaligenes eutrophus*) were used. The PHA synthase of this bacterium can utilize only short chain (C_3-C_5) monomers (Steinbuchel, A. (1991) *Biomaterials: Novel materials from biological materials*, D. Byrom, ed. (New York: Macmillan Publishers Ltd.), pp. 123-213).

Recently, the synthesis of PHA containing 3-hydroxyalkanoic acid monomers ranging from 6 to sixteen carbon in *Arabidopsis thaliana* was reported (Mittendorf *et al.* (1998) *Proc. Natl. Acad. Sci. USA 95*:13397-13402). To accumulate PHA, the *Arabidopsis* plants were transformed with a nucleotide sequence encoding PHA synthase from *Pseudomonas aeuginosa* that was modified for peroxisome targeting by the addition of a nucleotide sequence encoding the C-terminal 34 amino acids of a *Brassica napus* isocitrate lyase. In these plants, PHA was produced in glyoxysomes, leaf-type peroxisomes and vacuoles. However, PHA production was very low in the *Arabidopsis* plants, suggesting that either the introduced PHA synthase did not function properly in the intended organelle or more likely that the necessary substrates for the introduced PHA synthase were present at levels that were limiting for PHA synthesis. While this report demonstrated that PHA can be produced in peroxisomes of plants, the level of PHA produced in the plants was far below levels necessary for the commercial production of PHA in plants.

SUMMARY OF THE INVENTION

Methods are provided for producing PHA and intermediate molecules thereof in plants. The methods find use in the production of high-quality, biodegradable thermoplastics. The invention provides environmentally friendly alternatives to

petroleum-based methods for producing plastics. The methods involve genetically manipulating a plant to produce enzymes for PHA synthesis in its peroxisomes. The methods comprise stably integrating in the genome of a plant nucleotide sequences encoding enzymes involved in the synthesis of PHA, preferably PHA copolymers.

Also provided are plants, plant tissues, plant cells, and seeds thereof, that are genetically manipulated to produce at least one enzyme involved in the synthesis of PHA in plant peroxisomes.

Nucleotide molecules and expression cassettes comprising nucleotide sequences encoding enzymes that can be employed in the synthesis of PHA in the peroxisomes of plants are provided. In particular, the invention provides nucleotide molecules encoding a maize MFP2-like polypeptide, and fragments and variants thereof. Additionally, the invention provides nucleotide molecules comprising nucleotide sequences which encode for either the hydratase or the dehydrogenase domain of the yeast multifunctional protein-2 (MFP2). Such nucleotide molecules encode novel enzymes which find use in PHA synthesis in host cells and plants, particularly in peroxisomes of plants. Isolated polypeptides encoded by the nucleotide molecules of the invention and host cells transformed with such nucleotide molecules are additionally provided.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 schematically depicts possible biosynthetic steps for producing PHA in plant peroxisomes utilizing enzymes from bacteria.

Figure 2 schematically illustrates the full-length yeast multifunctional protein (yMFP), truncated versions of yMFP, and the maize MFP2-like polypeptide.

Figure 3 schematically depicts the biosynthetic pathways for the synthesis PHB and PHA copolymers which are composed of 3-hydroxybutanoic acid monomers and other 3-hydroxyalkanoate monomers.

Figure 4 schematically illustrates a functional assay for a 2-enoyl-CoA hydratase that catalyzes the synthesis of R-(-)-3-hydroxyacyl-CoA. This hydratase converts crotonyl-CoA to R-(-)-3-hydroxyacyl-CoA which is then utilized as the substrate by PHA synthase to form polymers. PHA synthase utiltizes R-(-)-3-hydroxyacyl-CoAs, but not S-3-hydroxyacyl-CoAs. Detection of polymer

formation in the presence of crotonyl-CoA demonstrates that R-(-)-3-hydroxyacyl-CoA was produced.

Figure 5 is a graphical depiction of the results of a functional assay for the activity of a 2-enoyl-CoA hydratase that catalyzes the synthesis R-(-)-3-hydroxyacyl-CoA.

DETAILED DESCRIPTION OF THE INVENTION

A number of terms used herein are defined and clarified in the following section.

By "PHA copolymer" is intended a polymer composed of at least two different 3-hydroxyalkanoic acid monomers.

By "PHA homopolymer" is intended a polymer that is composed of a single 3-hydroxyalkanoic acid monomer.

By "intermediate molecule" is intended a precursor in the biosynthetic pathway for PHA in a plant. Because PHA is not known to occur naturally in a plant, the biosynthetic pathway for PHA in plant additionally encompasses enzymes and products thereof that are involved in PHA synthesis which result from the genetic manipulation of the plant. Intermediate molecules of the present invention include, but are not limited to, fatty acids and β-oxidation products derived therefrom, acetyl-CoA, acetoacetyl-CoA and other 3-ketoacyl-CoAs, 3-hydroxybutyryl-CoA, and other 3-hydroxyacyl-CoAs.

By "modified or unusual" fatty acids is intended fatty acids that have structural features such as, for example, an epoxy group, a triple bond, and methyl branching. Such "modified or unusual" fatty acids include, but are not limited to, vernolic acid, petroselinic acid, sterculic acid, chaulmoogric acid, erucic acid, ricinoleic acid, labellenic acid, crepenynic acid, and stearolic acid.

The present invention is drawn to methods and compositions for producing PHA in plants. Particularly, the present invention provides improved methods for producing PHA in plant peroxisomes. The methods involve increasing the level of PHA produced in a plant by increasing the synthesis of at least one intermediate molecule in PHA synthesis. Thus, the methods involve modifying the metabolic functions of the peroxisome to allow for increased production of PHA in a plant.

Furthermore, the invention provides methods for producing PHA copolymers in plant peroxisomes.

Methods for producing PHB in the cytosol or plastids of plants and for producing PHA in plant peroxisomes are known in the art. However, such methods do not achieve the synthesis of high levels of PHA in plants. An object of the present invention is to provide improved methods for producing PHA, preferably PHA copolymers, in plants. The present invention involves genetically modifying plants in such a manner as to alter the metabolic functions of the peroxisome to increase the flux of carbon toward PHA synthesis. Such plants find use in preferred methods for producing high levels of PHA in plants, particularly in seeds, more particularly in oilseeds.

Methods for producing PHA in plants are provided. The methods involve genetically manipulating the genome of a plant to direct the synthesis of PHA to the peroxisomes, preferably peroxisomes in developing seeds. The invention encompasses plants and seeds thereof, that have been genetically manipulated to produce enzymes involved in PHA synthesis and expression cassettes containing coding sequences for such enzymes. The invention further encompasses genetically manipulated plant cells and plant tissues.

Peroxisomes, which are also known as microbodies, are small spherical organelles. In plants, there are generally two types of peroxisomes, leaf-type peroxisomes and glyoxysomes. Glyoxysomes are present in seeds containing oil, particularly during germination (Heldt (1997) *Plant Biochemistry and Plant Molecular Biology*, Oxford University Press, NY). In the present invention, "peroxisome" is intended to encompass all peroxisomes found in plant cells, including, but not limited to, leaf-type peroxisomes, microbodies, and glyoxysomes.

Methods are provided for producing PHA in a plant involving genetically manipulating the plant to produce in its peroxisomes at least two enzymes in the PHA biosynthetic pathway. The plants of the invention each comprise in their genomes at least two stably incorporated DNA constructs, each DNA construct comprising a coding sequence for an enzyme involved in PHA synthesis operably linked to a promoter that drives the expression of a gene in a plant. Plants of the invention are genetically manipulated to produce a PHA synthase (also known as a PHA polymerase) that catalyzes polymer synthesis. Preferably, such a PHA synthase

catalyzes the synthesis of copolymers. More preferably such a PHA synthase catalyzes the synthesis of copolymers comprised of 3-hydroxybutanoic acid monomers and at least one additional monomer having a chain length of greater than four carbons. Most preferably such a PHA synthase catalyzes the synthesis of copolymers comprised of 3-hydroxybutanoic acid monomers and at least one additional monomer having a hydroxyacyl-chain length of from about 5 to about 18 carbons. Preferred PHA synthases include PHA synthases encoded by nucleotide sequences isolatable from *Pseudomonas oleovorans* (GenBank Accession No. M58445, SEQ ID NO: 8), *Pseudomonas putida* (GenBank Accession No. AF042276, SEQ ID NO: 9), *Pseudomonas aeruginosa* (EMBL Accession No. X66592, SEQ ID NO: 10), *Aeromonas caviae* (DDBJ Accession No. D88825, SEQ ID NO: 11), and *Thiocapsa pfennigii* (EMBL Accession No. A49465, SEQ ID NO: 12). The preferred PHA synthases additionally include the PHA synthases encoded by nucleotide sequences isolatable from *Pseudomonas fluorescens* (See U.S. Provisional Patent Application No. 60/156,770 filed September 29, 1999; herein incorporated by reference.). In certain methods of the invention, the majority of PHA copolymers produced are comprised of monomers of chain-length C_4 to C_{18}.

The DNA constructs of the invention each comprise a coding sequence for an enzyme involved in PHA synthesis operably linked to a promoter that drives expression in a plant cell. Preferably, the promoters are selected from seed-preferred promoters, chemical-regulatable promoters, germination-preferred promoters, and leaf-preferred promoters. If necessary for directing the encoded proteins to the peroxisome, the DNA construct can include an operably linked peroxisome-targeting signal sequence.

It is recognized that for producing high levels of PHA copolymers in certain plants, particularly in their peroxisomes, it may be necessary to genetically manipulate plants to produce additional enzymes involved in PHA synthesis. Generally, the additional enzymes are directed to the peroxisome to increase the synthesis of at least one intermediate molecule. For example, such an intermediate molecule can be the substrate for a PHA synthase including, but not limited to, an R-(-)-3-hydroxyacyl-CoA. The methods of the invention comprise genetically modifying plants to produce, in addition to the PHA synthase described *supra*, one, two, three, four, or more additional enzymes involved in PHA synthesis. Preferably,

each DNA construct comprising the coding sequence of one of these additional enzymes is operably linked to a promoter that drives expression in a plant and also to a nucleotide sequence encoding a peroxisome-targeting signal sequence. Depending on the plant, the addition of one or more of these enzymes may be necessary to achieve high-level PHA synthesis in the plant. The additional enzymes include, but are not limited to, an enzyme that catalyzes the synthesis of R-(-)-3-hydroxyacyl-CoA, a 3-ketoacyl-CoA reductase, and an acetyl-CoA:acetyl transferase.

Additionally, the plant of the invention can comprise in its genome a DNA construct comprising a coding sequence for second PHA synthase. Preferably, the second PHA synthase is capable of synthesizing PHB. Preferred second PHA synthases include those encoded by nucleotide sequences isolatable *from Ralstonia eutropha* (GenBank Accession No. J05003, SEQ ID NO: 13), *Acinetobacter sp.* (GenBank Accession No. U04848, SEQ ID NO: 14), *Alcaligenes latus* (GenBank Accession No. AF078795, SEQ ID NO: 15), *Azorhizobium caulinodans* (EMBL Accession No. AJ006237, SEQ ID NO: 16), *Comamonas acidovorans* (DDBJ Accession No. AB009273, SEQ ID NO: 17), *Methylobacterium extorquens* (GenBank Accession No. L07893, SEQ ID NO: 18), *Paracoccus denitrificans* (DDBJ Accession No. D43764, SEQ ID NO: 19), and *Zoogloea ramigera* (GenBank U66242, SEQ ID NO: 20)

The methods of the invention additionally comprise growing the plant under conditions favorable for PHA production, harvesting the plant, or one or more parts thereof, and isolating the PHA from the plant or part thereof. Such parts include, but are not limited to, seeds, leaves, stems, roots, fruits, and tubers. The PHA can be isolated from the plant or part thereof by methods known in the art. See, U.S. Patent Nos. 5,942,597; 5,918,747; 5,899,339; 5,849,854; and 5,821,299; herein incorporated by reference. See also, EP 859858A1, WO 97/07229, WO 97/07230, and WO 97/15681; herein incorporated by reference.

The invention provides methods for producing increased levels of PHA in the peroxisomes of plants that involve increasing the synthesis of one or more intermediate molecules in the peroxisome. The methods involve diverting the flux of carbon in the peroxisome to favor PHA synthesis over endogenous metabolic processes such as, for example, β-oxidation. In a first aspect of the invention, plants

are genetically manipulated to increase the synthesis of R-(-)-3-hydroxyacyl-CoAs. In a second aspect of the invention, plants are genetically manipulated to increase the synthesis of a specific R-(-)-3-hydroxyacyl-CoA, R-(-)-3-hydroxybutyryl-CoA. In a third aspect of the invention, the first and second aspects are combined to provide plants that are genetically manipulated to increase the synthesis of both R-(-)-3-hydroxyacyl-CoAs and R-(-)-3-hydroxybutyryl-CoA.

Further, it is recognized that each of the aspects of the invention can be used to produce PHA with substantially different monomer compositions. In particular, the level of 3-hydroxybutanoic acid in the PHA produced in a plant will vary with each aspect. For each particular type of plant, PHA produced by plants of the second or third aspect of the invention is expected to have a higher 3-hydroxybutanoic acid monomer content than PHA produced by plants of the first aspect. Similarly, PHA produced by plants of the second aspect is expected to have a higher 3-hydroxybutanoic acid monomer content than PHA produced by plants of the third aspect.

In a first embodiment of the invention, methods are provided for producing PHA involving genetically manipulating a plant for increased synthesis of R-(-)-3-hydroxyacyl-CoA, a key intermediate molecule in PHA synthesis in the peroxisome. The methods comprise stably integrating into the genome of a plant a first DNA construct comprising a coding sequence for a PHA synthase, and a second DNA construct comprising a coding sequence for an enzyme that catalyzes the formation R-(-)-3-hydroxyacyl-CoA, a substrate of PHA synthase. In β-oxidation in plant peroxisomes, acyl-CoA oxidase catalyzes the conversion of fatty acyl-CoA into 2- enoyl-CoA which is subsequently converted to S-(+)-3-hydroxyacyl-CoA via the 2-enoyl-CoA hydratase of a multifunctional protein. While some R-(-)-3-hydroxyacyl-CoA may be present in peroxisomes, the level is believed to be very low and insufficient to allow for the synthesis of an economically acceptable level of PHA in a plant. Furthermore, all known PHA synthases require that 3-hydroxyacyl-CoA monomers be in R-(-)-form for PHA synthesis. To overcome the substrate limitation for PHA synthesis, the present invention discloses methods for PHA synthesis which involve providing a plant with an enzyme in its peroxisomes that catalyzes the formation of R(-)-3-hydroxyacyl-CoA. By genetically manipulating a plant to increase the synthesis of R-(-)-3-hydroxyacyl-CoA, the present invention

overcomes a major impediment to achieving high-level production in plants of PHA, particularly copolymers. Such an enzyme can be an enoyl-CoA hydratase that catalyzes the synthesis of R-(-)-3-hydroxyacyl-CoA, particularly an 2-enoyl-CoA hydratase from *Aeromonas caviae* (DDBJ Accession No. E15860, SEQ ID NO: 21).

Alternatively, two proteins from yeast and one from maize can each be utilized as the enzyme. One such protein is the yeast multifunctional protein (encoded by GenBank Accession No. M86456, SEQ ID NO: 3) which possesses an 2-enoyl-CoA hydratase activity and a 3-hydroxyacyl dehydrogenase (reductase) activity. Similarly, the invention provides an isolated MFP2-like polypeptide (previously designated as a multifunctional protein-2 or MFP-2) from maize (SEQ ID NO: 2) which also possesses an 2-enoyl-CoA hydratase activity. The invention further provides isolated nucleotide molecules encoding such a maize MFP2-like polypeptide (SEQ ID NO: 1). The hydratase of the yeast multifunctional protein and maize MFP2-like polypeptide is known to yield R-(-)-3-hydroxyacyl-CoA products. If necessary, the dehydrogenase activity of the yeast multifunctional protein can be eliminated or neutralized by methods known to those of ordinary skill in the art such as, for example, site-directed mutagenesis, and truncation of the coding sequence to only the portion necessary to encode the desired hydratase activity. The invention provides isolated nucleotide molecules comprising nucleotide sequences which encode either the hydratase or reductase of the yeast multifunctional protein (SEQ ID NOs: 4 and 6). Additionally provided are isolated polypeptides encoded by such sequences (SEQ ID NOs: 5 and 7).

Other multifunctional proteins known in the art can be utilized in the methods of the present invention. Any multifunctional protein possessing a domain comprising a 2-enoyl-CoA hydratase that is capable of catalyzing the synthesis of R-(-)-3-hydroxyacyl-CoA can be employed in the methods of the invention.

The other yeast protein that can be utilized as the enzyme that catalyzes the formation of R-(-)-3-hydroxyacyl-CoA is an enzyme identified as a 3-hydroxybutyryl-CoA dehydrogenase (Leaf *et al.* (1996) *Microbiology 142*:1169-1180). The gene encoding this enzyme can be cloned from *Saccharomyces cervisae*, sequenced and employed in the methods of the present invention. It is recognized that the nucleotide sequence encoding this enzyme can be modified to alter the amino acid sequence of the enzyme in such a manner as to favorably affect the production of

R-(-)-3-hydroxyacyl-CoA in a plant. Such modifications can affect characteristics of the enzyme such as, for example, substrate specificity, product specificity, product inhibition, substrate binding affinity, product binding affinity, and the like. A method such as, for example, DNA shuffling can be employed to modify this enzyme in the desired manner. Any method known in the art for altering the characteristics of an enzyme to favorably affect the mass action ratio toward the desired product is encompassed by the methods of the present invention. Such methods typically involve modifying at least a portion of the nucleotide sequence encoding the enzyme and include, but are not limited to, DNA shuffling, site-directed mutagenesis, and random mutagenesis.

In a second embodiment of the invention, methods are provided for producing PHA involving genetically manipulating a plant for increased synthesis of R-(-)-3-hydroxybutyryl-CoA, a substrate of PHA synthase, in peroxisomes. The methods of the invention provide a plant that is genetically manipulated for increased synthesis of a substrate for a PHA synthase and thus provide a plant that is genetically manipulated for high-level PHA synthesis in its peroxisomes. The methods involve stably integrating into the genome of a plant a first DNA construct comprising a coding sequence for a PHA synthase, and a second DNA construct comprising a coding sequence for 3-ketoacyl-CoA reductase and a third DNA construct comprising a coding sequence for an acetyl-CoA:acetyl transferase. The first, second, and third DNA constructs each additionally comprise an operably linked promoter that drives expression in a plant cell, and if necessary, an operably linked peroxisome-targeting signal sequence. Acetyl-CoA:acetyl transferase, also referred to as ketothiolase, catalyzes the synthesis of acetoacetyl-CoA from two molecules of acetyl-CoA. Acetoacetyl-CoA can then be converted into R-(-)-3-hydroxybutyryl-CoA *via* a reaction catalyzed by a 3-ketoacyl-CoA reductase, particularly an acetoacetyl-CoA reductase.

Preferred 3-ketoacyl-CoA reductases of the invention are those that utilize NADH and include, but are not limited to, at least a portion of the multifunctional proteins from yeast (encoded by GenBank Accession No. M86456, SEQ ID NO: 3) and rat (encoded by GenBank Accession No. U37486, SEQ ID NO: 22), wherein such a portion comprises a 3-ketoacyl-CoA reductase domain. Any multifunctional protein having a 3-ketoacyl-CoA reductase (dehydrogenase) domain can be employed in the

methods of the invention. However, in the methods of the invention, NADPH-dependent 3-ketoacyl-CoA reductases can also be employed including, but not limited to, the 3-ketoacyl-CoA reductases encoded by GenBank Accession No. J04987 (SEQ ID NO: 23).

Preferred acetyl-CoA:acetyl transferases of the invention include a radish acetyl-CoA:acetyl transferase encoded by the nucleotide sequence having EMBL Accession No. X78116 (SEQ ID NO: 24).

If necessary to increase the level of NADPH in the peroxisome, the methods of the second embodiment can additionally involve, stably integrating into the genome of a plant a fourth DNA construct comprising a nucleotide sequence encoding an NADH kinase or an NAD^+ kinase and an operably linked promoter that drives expression in a plant cell. Such NADH and NAD^+ kinases catalyze the synthesis of NADPH and $NADP^+$, respectively. Nucleotide sequences encoding such kinases include, but are not limited to, DDJB Accession No. E13102 (SEQ ID NO: 25) and EMBL Accession Nos. Z73544 (SEQ ID NO: 26) and X84260 (SEQ ID NO: 27). The fourth construct can additionally comprise an operably linked peroxisome-targeting signal sequence. By targeting such NADH and NAD^+ kinases to the peroxisome, the level of NADPH and $NADP^+$ can be increased in the plant peroxisome for use by enzymes, such as, for example, an NADPH-dependent 3-ketoacyl-CoA reductase.

In a third embodiment of the invention, methods are provided for producing PHA in a plant involving genetically manipulating a plant for increased synthesis of R-(-)-3-hydroxybutyryl-CoA and other R-(-)-3-hydroxyacyl-CoA molecules. Such methods provide a plant that is genetically manipulated to overcome substrate limitations for PHA copolymer synthesis in its peroxisomes. The methods involve stably integrating into the genome of a plant a first, a second, a third, and a fourth DNA construct comprising a coding sequence for an enzyme involved in PHA synthesis in a plant. The first DNA construct comprises a coding sequence for a PHA synthase that is capable of catalyzing the synthesis of PHA copolymers. The second DNA construct comprises a coding sequence for an enzyme that catalyzes the synthesis of R-(-)-3-hydroxyacyl-CoA. The third DNA construct comprises a coding sequence for a 3-ketoacyl-CoA reductase, and the fourth DNA construct comprises a coding sequence for an acetyl-CoA:acetyl transferase. If desired, a fifth DNA

construct can also be stably integrated into the genome of the plant. The fifth DNA construct comprises a nucleotide sequence encoding a NADH kinase or an NAD^+ kinase.

Preferred enzymes of the third embodiment include the enzymes of the first and second embodiments, described *supra*. The DNA constructs each additionally comprise an operably linked promoter and, if necessary, an operably linked peroxisome-targeting signal to direct the encoded protein to the peroxisome. By targeting such enzymes to the peroxisome, the plant is capable of increased synthesis of intermediate molecules, particularly intermediate molecules that are substrates for a PHA synthase that catalyzes the formation of copolymers.

Methods are provided for increasing the synthesis of PHA in a plant. Such methods find use with methods known in the art for producing PHA in plants, particularly in peroxisomes. The methods of the invention involve increasing the synthesis of an intermediate molecule in PHA synthesis. Preferably, such an intermediate molecule is limiting for PHA synthesis in the peroxisome and that increasing the synthesis of such a molecule in the peroxisome increases the level of PHA produced in a plant. It is recognized that increasing the synthesis of an intermediate molecule in a plant peroxisome might not lead to an increased level of the intermediate molecule in the plant because the intermediate molecule can be further metabolized into, for example, PHA.

The methods for increasing the synthesis of PHA in a plant involve stably incorporating into the genome of a plant at least one DNA construct comprising a coding sequence for an enzyme involved in the synthesis of an intermediate molecule, operably linked to a promoter that drives expression in a plant. If necessary for peroxisome-targeting of the encoded enzyme, the DNA construct additionally comprises a peroxisome-targeting signal operably linked to the coding sequence.

The methods of the invention can be used to increase the synthesis of any intermediate molecule in PHA synthesis. Preferred intermediate molecules include those that can be limiting for PHA synthesis, particularly in the peroxisome, such as, for example, R-(-)-3-hydroxybutyryl-CoA, other R-(-)-3-hydroxyacyl-CoAs, acetoacetyl-CoA, and other 3-ketoacyl-CoAs.

A plant can be genetically manipulated to produce any one or more of the enzymes involved in the synthesis of the intermediate molecule in the plant including,

but not limited to, the enzymes for PHA synthesis of the present invention described *supra*. Preferred enzymes for increasing the synthesis of an intermediate molecule include enzymes, described *supra*, that catalyze the formation of R-(-)-3-hydroxyacyl-CoA, 3-ketoacyl-CoA reductases that utilize NADH and acetyl-CoA:acetyl transferases.

In a fourth embodiment of the invention, methods are provided for increasing in a plant the synthesis of a R-(-)-3-hydroxyacyl-CoA, key intermediate molecule in PHA synthesis. The level in the peroxisome of R-(-)-3-hydroxyacyl-CoA, a substrate of PHA synthase, is known to be very low and is believed to limit the level of PHA produced in the peroxisome. Thus, increasing the synthesis of R-(-)-3-hydroxyacyl-CoA can increase the synthesis of PHA in a plant. The methods comprise genetically manipulating plants to produce an enzyme that catalyzes the synthesis of R-(-)-3-hydroxyacyl-CoA, preferably an enzyme selected from a 2-enoyl-CoA hydratase from *Aeromonas caviae* (encoded by DDBJ Accession No. E15860, SEQ ID NO: 21), a maize MFP2-like polypeptide (SEQ ID NO: 2), a modified yeast multifunctional protein (SEQ ID NO: 5) possessing a 2-enoyl-CoA hydratase activity and a 3-hydroxyacyl-CoA dehydrogenase activity wherein the latter activity is neutralized, or a modified yeast 3-hydroxyacyl-CoA dehydrogenase, wherein the enzyme utilizes NADH and its mass action ratio has been shifted in favor of R-(-)-3-hydroxyacyl-CoA over 3-ketoacyl-CoA.

Methods are provided for producing novel enzymes for the synthesis of PHA, particularly in peroxisomes, more particularly in plant peroxisomes. The methods find use in providing novel peroxisome-localized enzymes for PHA synthesis in plant peroxisomes. Additionally, the methods find further use in providing DNA constructs that can be used to transform a plant for the production of a PHA synthesis enzyme in its peroxisomes. The methods involve modifying the coding sequence of a multifunctional protein, particularly an MFP2, more particularly an MFP2 from yeast (encoded by GenBank Accession No. M86456, SEQ ID NO: 3) or rat (encoded by GenBank Accession No. U37486, SEQ ID NO: 22). The coding sequence is modified to eliminate or substantially reduce of one of the two separate enzymatic activities of the protein encoded thereby. The coding sequence can be modified by methods known in the art including, but not limited, to site-directed mutagenesis and deletion of a portion of the coding sequence. If necessary, an initiation codon, a stop codon or

both can be added to facilitate translation in a host cell. The methods can further involve preparing a DNA construct by operably linking a nucleotide sequence encoding a peroxisome-targeting signal to the modified coding sequence. If desired for expression in a plant or host cell, the DNA construct can additionally comprise an operably linked promoter that drives expression in the plant or cell. The novel enzymes of the invention can be produced by transforming a plant or host cell with such a DNA construct.

In a fifth embodiment of the invention, methods are provided for producing a peroxisome-targeted 2-enoyl-CoA hydratase employing the coding sequence of a yeast MFP2 (GenBank Accession No. M86456, SEQ ID NO: 3). The methods involve modifying the coding sequence to eliminate the dehydrogenase (also referred to as reductase) activity of the encoded protein. This can be accomplished by, for example, deleting from the 5' end of the coding sequence a portion that encoded at least part of the dehydrogenase domain (Figure 2, SEQ ID NO: 4). To facilitate translation of the encoded protein, an initiation codon can be added to the truncated nucleotide sequence (SEQ ID NO: 4). A DNA construct can be prepared by operably linking a nucleotide sequence encoding a plant peroxisome-targeting signal to such a truncated coding sequence. Such a DNA construct can additionally comprise an operably linked promoter that drives expression in a plant.

In a sixth embodiment of the invention, methods are provided for producing a NADH-dependent, peroxisome-targeted 3-ketoacyl-CoA reductase employing the coding sequence of a yeast multifunctional protein (GenBank Accession No. M86456, SEQ ID NO: 3). The methods involve modifying the coding sequence of a multifunctional protein to eliminate the hydratase activity of the encoded protein. Because the reductase of the yeast multifunctional protein is known to be NADH dependent, a peroxisome-targeted 3-ketoacyl-CoA reductase is expected to be NADH dependent. Such an NADH-dependent reductase finds use in the high-level synthesis of PHA in plant peroxisomes where NADPH, but not NADH, is known to be limiting. This can be accomplished by, for example, deleting from the 3' end of the coding sequence a portion that encoded at least a part of the hydratase domain (Figure 2, SEQ ID NO: 6). To facilitate translation of the desired polypeptide, an appropriate stop codon can be operably linked to the 3' end of the truncated coding sequence (SEQ ID NO: 6). In addition, a DNA construct can be prepared by operably linking a

nucleotide sequence encoding a plant peroxisome-targeting signal to such a truncated coding sequence. Such a DNA construct can additionally comprise an operably linked promoter that drives expression in a plant.

Methods are provided for increasing the level in a plant of at least one intermediate molecule in PHA synthesis in the plant. Such intermediate molecules include molecules naturally synthesized by the plant as well as those that are synthesized by the plant after being genetically manipulated to comprise at least one enzyme involved in PHA synthesis that does not occur naturally in the plant. The methods involve the buildup of intermediate molecules as well as the use of additional enzymes for the production of specialty chemicals. Thus, plants containing intermediate molecules or PHA can be obtained. It is recognized that methods of the present invention can be used in combination with methods for producing PHA homopolymers, copolymers or both.

To increase the level of at least one intermediate molecule in a plant, the plant can be genetically manipulated to produce any one or more of the enzymes involved in the synthesis of the intermediate molecule in the plant including, but not limited to, the enzymes for PHA synthesis of the present invention described *supra*. Preferred enzymes for increasing the synthesis of an intermediate molecule include enzymes, described *supra*, that catalyze the formation of R-(-)-3-hydroxyacyl-CoA, 3-ketoacyl-CoA reductases that utilize NADH and acetyl-CoA:acetyl transferases.

Further, it is recognized that it may be necessary to lower or eliminate the activity of an endogenous enzyme in a plant that in some way limits the synthesis of the desired intermediate molecule. Such an endogenous enzyme may, for example, catabolize or modify the intermediate molecule in an undesirable way. Methods for lowering or eliminating the activity of an enzyme in a plant include, but are not limited to, sense and antisense suppression methods. For example, the activity of the 2-enoyl-CoA hydratase of an endogenous multifunctional protein that catalyzes the formation of S-(+)-3-hydroxyacyl-CoA can be reduced or eliminated in the peroxisome to favor, instead, the synthesis of R-(-)-3-hydroxyacyl-CoA therein.

While the methods of the invention can be used with any plant, preferred plants are oilseed plants genetically manipulated to produce PHA copolymers in their peroxisomes, particularly in seeds. More preferably, the oilseed plants have been genetically manipulated to have seeds with increased levels of short-chain fatty acids,

modified or unusual fatty acids, cytosolic acyl-CoA oxidase activity, or combinations thereof. Such oilseed plants have increased rates of β-oxidation in their seeds and find use in methods of producing high levels of PHA copolymers, particularly in seeds.

While the compositions and methods disclosed herein are drawn to the production of PHA and PHA intermediates in plants, the present invention is not limited to methods involving PHA production in plant and cells thereof. Those skilled in the art will recognize that the compositions and methods can be employed with any host cell for the production of PHA and intermediates thereof. Host cells include, but are not limited to, plant cells, animal cells, bacterial cells and fungal cells, particularly yeast cells.

Compositions comprising nucleic acid molecules which comprise coding sequences for enzymes involved in the synthesis of PHA in the peroxisomes of plants are provided. Compositions of the invention include nucleotide molecules encoding a maize MFP2-like polypeptide and fragments and variants thereof. In particular, the present invention provides for isolated nucleic acid molecules comprising nucleotide sequences encoding the amino acid sequence set forth in SEQ ID NO: 2. Further provided are polypeptides having an amino acid sequence encoded by a nucleic acid molecule described herein, for example that set forth in SEQ ID NO: 1, and fragments and variants thereof.

The invention encompasses isolated or substantially purified nucleic acid or protein compositions. An "isolated" or "purified" nucleic acid molecule or protein, or biologically active portion thereof, is substantially free of other cellular material, or culture medium when produced by recombinant techniques, or substantially free of chemical precursors or other chemicals when chemically synthesized. Preferably, an "isolated" nucleic acid is free of sequences (preferably protein encoding sequences) that naturally flank the nucleic acid (i.e., sequences located at the 5' and 3' ends of the nucleic acid) in the genomic DNA of the organism from which the nucleic acid is derived. For example, in various embodiments, the isolated nucleic acid molecule can contain less than about 5 kb, 4 kb, 3 kb, 2 kb, 1 kb, 0.5 kb, or 0.1 kb of nucleotide sequences that naturally flank the nucleic acid molecule in genomic DNA of the cell from which the nucleic acid is derived. A protein that is substantially free of cellular material includes preparations of protein having less than about 30%, 20%, 10%, 5%,

(by dry weight) of contaminating protein. When the protein of the invention or biologically active portion thereof is recombinantly produced, preferably culture medium represents less than about 30%, 20%, 10%, or 5% (by dry weight) of chemical precursors or non-protein-of-interest chemicals.

Fragments and variants of the disclosed nucleotide sequences and proteins encoded thereby are also encompassed by the present invention. By "fragment" is intended a portion of the nucleotide sequence or a portion of the amino acid sequence and hence protein encoded thereby. Fragments of a nucleotide sequence may encode protein fragments that retain the biological activity of the native protein of the invention. Alternatively, fragments of a nucleotide sequence that are useful as hybridization probes generally do not retaining biological activity. Thus, fragments of a nucleotide sequence may range from at least about 20 nucleotides, about 50 nucleotides, about 100 nucleotides, and up to the full-length nucleotide sequence encoding the proteins of the invention.

A fragment of a nucleotide sequence of the invention that encodes a biologically active portion of a multifunctional protein will encode at least 15, 25, 30, 50, 100, 150, 200, 250 or 300 contiguous amino acids, or up to the total number of amino acids present in a full-length multifunctional protein of the invention (for example, 314 amino acids for SEQ ID NO: 2). Fragments of a multifunctional protein nucleotide sequence that are useful as hybridization probes for PCR primers generally need not encode a biologically active portion of a multifunctional protein.

Thus, a fragment of a multifunctional protein nucleotide sequence may encode a biologically active portion of a multifunctional protein, or it may be a fragment that can be used as a hybridization probe or PCR primer using methods disclosed below. A biologically active portion of a multifunctional protein can be prepared by isolating a portion of one of the multifunctional protein nucleotide sequences of the invention, expressing the encoded portion of the multifunctional protein (e.g., by recombinant expression *in vitro*), and assessing the activity of the encoded portion of the multifunctional protein. Nucleic acid molecules that are fragments of a multifunctional protein nucleotide sequence comprise at least 16, 20, 50, 75, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 800, 900, 1,000, 1,100, 1,200 or 1,300 nucleotides, or up to the number of nucleotides present in a full-length

multifunctional protein nucleotide sequence disclosed herein (for example, 1362 nucleotides for SEQ ID NO: 1).

By "variants" is intended substantially similar sequences. For nucleotide sequences, conservative variants include those sequences that, because of the degeneracy of the genetic code, encode the amino acid sequence of one of the multifunctional proteins or other enzymes involved in PHA synthesis of the invention. Naturally occurring allelic variants such as these can be identified with the use of well-known molecular biology techniques, as, for example, with polymerase chain reaction (PCR) and hybridization techniques as outlined below. Variant nucleotide sequences also include synthetically derived nucleotide sequences, such as those generated, for example, by using site-directed mutagenesis but which still encode a multifunctional protein or other enzyme of the invention. Generally, variants of a particular nucleotide sequence of the invention will have at least about 50%, 60%, 65%, 70%, generally at least about 75%, 80%, 85%, preferably at least about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, and more preferably at least about 98%, 99% or more sequence identity to that particular nucleotide sequence as determined by sequence alignment programs described elsewhere herein using default parameters.

The nucleotide sequences of the invention can be used to isolate corresponding sequences from other organisms, particularly other plants, more particularly other monocots. In this manner, methods such as PCR, hybridization, and the like can be used to identify such sequences based on their sequence homology to the sequence set forth herein. Sequences isolated based on their sequence identity to the entire maize MFP2-like polypeptide nucleotide sequence set forth herein or to fragments thereof are encompassed by the present invention. Such sequences include sequences that are orthologs of the disclosed sequences. By "orthologs" is intended genes derived from a common ancestral gene and which are found in different species as a result of speciation. Genes found in different species are considered orthologs when their nucleotide sequences and/or their encoded protein sequences share substantial identity as defined elsewhere herein. Functions of orthologs are often highly conserved among species.

In a PCR approach, oligonucleotide primers can be designed for use in PCR reactions to amplify corresponding DNA sequences from cDNA or genomic DNA extracted from any organism of interest. Methods for designing PCR primers and

PCR cloning are generally known in the art and are disclosed in Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York). See also Innis *et al.*, eds. (1990) *PCR Protocols: A Guide to Methods and Applications* (Academic Press, New York); Innis and Gelfand, eds. (1995) *PCR Strategies* (Academic Press, New York); and Innis and Gelfand, eds. (1999) *PCR Methods Manual* (Academic Press, New York). Known methods of PCR include, but are not limited to, methods using paired primers, nested primers, single specific primers, degenerate primers, gene-specific primers, vector-specific primers, partially-mismatched primers, and the like.

In hybridization techniques, all or part of a known nucleotide sequence is used as a probe that selectively hybridizes to other corresponding nucleotide sequences present in a population of cloned genomic DNA fragments or cDNA fragments (i.e., genomic or cDNA libraries) from a chosen organism. The hybridization probes may be genomic DNA fragments, cDNA fragments, RNA fragments, or other oligonucleotides, and may be labeled with a detectable group such as ^{32}P, or any other detectable marker. Thus, for example, probes for hybridization can be made by labeling synthetic oligonucleotides based on the maize MFP2-like polypeptide nucleotide sequence of the invention. Methods for preparation of probes for hybridization and for construction of cDNA and genomic libraries are generally known in the art and are disclosed in Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York).

For example, the entire maize MFP2-like polypeptide nucleotide sequence disclosed herein, or one or more portions thereof, may be used as a probe capable of specifically hybridizing to corresponding MFP2 sequences and messenger RNAs. To achieve specific hybridization under a variety of conditions, such probes include sequences that are unique among MFP2 sequences and are preferably at least about 10 nucleotides in length, and most preferably at least about 20 nucleotides in length. Such probes may be used to amplify corresponding MFP2 sequences from a chosen organism by PCR. This technique may be used to isolate additional coding sequences from a desired organism or as a diagnostic assay to determine the presence of coding sequences in a organism. Hybridization techniques include hybridization screening of plated DNA libraries (either plaques or colonies; see, for example, Sambrook *et al.*

(1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York).

Hybridization of such sequences may be carried out under stringent conditions. By "stringent conditions" or "stringent hybridization conditions" is intended conditions under which a probe will hybridize to its target sequence to a detectably greater degree than to other sequences (e.g., at least 2-fold over background). Stringent conditions are sequence-dependent and will be different in different circumstances. By controlling the stringency of the hybridization and/or washing conditions, target sequences that are 100% complementary to the probe can be identified (homologous probing). Alternatively, stringency conditions can be adjusted to allow some mismatching in sequences so that lower degrees of similarity are detected (heterologous probing). Generally, a probe is less than about 1000 nucleotides in length, preferably less than 500 nucleotides in length.

Typically, stringent conditions will be those in which the salt concentration is less than about 1.5 M Na ion, typically about 0.01 to 1.0 M Na ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes (e.g., 10 to 50 nucleotides) and at least about 60°C for long probes (e.g., greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. Exemplary low stringency conditions include hybridization with a buffer solution of 30 to 35% formamide, 1 M NaCl, 1% SDS (sodium dodecyl sulphate) at 37°C, and a wash in 1X to 2X SSC (20X SSC = 3.0 M NaCl/0.3 M trisodium citrate) at 50 to 55°C. Exemplary moderate stringency conditions include hybridization in 40 to 45% formamide, 1.0 M NaCl, 1% SDS at 37°C, and a wash in 0.5X to 1X SSC at 55 to 60°C. Exemplary high stringency conditions include hybridization in 50% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 0.1X SSC at 60 to 65°C. The duration of hybridization is generally less than about 24 hours, usually about 4 to about 12 hours.

Specificity is typically the function of post-hybridization washes, the critical factors being the ionic strength and temperature of the final wash solution. For DNA-DNA hybrids, the T_m can be approximated from the equation of Meinkoth and Wahl (1984) *Anal. Biochem. 138*:267-284: $T_m = 81.5°C + 16.6 (\log M) + 0.41 (\%GC) - 0.61 (\%\ form) - 500/L$; where M is the molarity of monovalent cations, %GC is the percentage of guanosine and cytosine nucleotides in the DNA, % form is the

percentage of formamide in the hybridization solution, and L is the length of the hybrid in base pairs. The T_m is the temperature (under defined ionic strength and pH) at which 50% of a complementary target sequence hybridizes to a perfectly matched probe. T_m is reduced by about 1°C for each 1% of mismatching; thus, T_m, hybridization, and/or wash conditions can be adjusted to hybridize to sequences of the desired identity. For example, if sequences with ≥90% identity are sought, the T_m can be decreased 10°C. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequence and its complement at a defined ionic strength and pH. However, severely stringent conditions can utilize a hybridization and/or wash at 1, 2, 3, or 4°C lower than the thermal melting point (T_m); moderately stringent conditions can utilize a hybridization and/or wash at 6, 7, 8, 9, or 10°C lower than the thermal melting point (T_m); low stringency conditions can utilize a hybridization and/or wash at 11, 12, 13, 14, 15, or 20°C lower than the thermal melting point (T_m). Using the equation, hybridization and wash compositions, and desired T_m, those of ordinary skill will understand that variations in the stringency of hybridization and/or wash solutions are inherently described. If the desired degree of mismatching results in a T_m of less than 45°C (aqueous solution) or 32°C (formamide solution), it is preferred to increase the SSC concentration so that a higher temperature can be used. An extensive guide to the hybridization of nucleic acids is found in Tijssen (1993) *Laboratory Techniques in Biochemistry and Molecular Biology—Hybridization with Nucleic Acid Probes*, Part I, Chapter 2 (Elsevier, New York); and Ausubel *et al.*, eds. (1995) *Current Protocols in Molecular Biology*, Chapter 2 (Greene Publishing and Wiley-Interscience, New York). See Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York).

Thus, isolated sequences that encode for an MFP2 and which hybridize under stringent conditions to the MFP2 sequence disclosed herein, or to fragments thereof, are encompassed by the present invention. Such sequences will be at least about 50% to 60% homologous, about 60%, to 70% homologous, and even at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more homologous with the disclosed sequences. That is, the sequence identity of sequences may range, sharing at least about 50% to 60%, about 65% or 70%, and even at least

about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity.

The following terms are used to describe the sequence relationships between two or more nucleic acids or polynucleotides: (a) "reference sequence", (b) "comparison window", (c) "sequence identity", (d) "percentage of sequence identity", and (e) "substantial identity".

(a) As used herein, "reference sequence" is a defined sequence used as a basis for sequence comparison. A reference sequence may be a subset or the entirety of a specified sequence; for example, as a segment of a full-length cDNA or gene sequence, or the complete cDNA or gene sequence.

(b) As used herein, "comparison window" makes reference to a contiguous and specified segment of a polynucleotide sequence, wherein the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Generally, the comparison window is at least 20 contiguous nucleotides in length, and optionally can be 30, 40, 50, 100, or longer. Those of skill in the art understand that to avoid a high similarity to a reference sequence due to inclusion of gaps in the polynucleotide sequence a gap penalty is typically introduced and is subtracted from the number of matches.

Methods of alignment of sequences for comparison are well known in the art. Thus, the determination of percent sequence identity between any two sequences can be accomplished using a mathematical algorithm. Non-limiting examples of such mathematical algorithms are the algorithm of Myers and Miller (1988) *CABIOS 4*:11-17; the local homology algorithm of Smith *et al.* (1981) *Adv. Appl. Math. 2*:482; the homology alignment algorithm of Needleman and Wunsch (1970) *J. Mol. Biol. 48*:443-453; the search-for-similarity-method of Pearson and Lipman (1988) *Proc. Natl. Acad. Sci. 85*:2444-2448; the algorithm of Karlin and Altschul (1990) *Proc. Natl. Acad. Sci. USA 872264*, modified as in Karlin and Altschul (1993) *Proc. Natl. Acad. Sci. USA 90*:5873-5877.

Computer implementations of these mathematical algorithms can be utilized for comparison of sequences to determine sequence identity. Such implementations include, but are not limited to: CLUSTAL in the PC/Gene program (available from Intelligenetics, Mountain View, California); the ALIGN program (Version 2.0) and

GAP, BESTFIT, BLAST, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Version 8 (available from Genetics Computer Group (GCG), 575 Science Drive, Madison, Wisconsin, USA). Alignments using these programs can be performed using the default parameters. The CLUSTAL program is well described by Higgins *et al.* (1988) *Gene 73*:237-244 (1988); Higgins *et al.* (1989) *CABIOS 5*:151-153; Corpet *et al.* (1988) *Nucleic Acids Res. 16*:10881-90; Huang *et al.* (1992) *CABIOS 8*:155-65; and Pearson *et al.* (1994) *Meth. Mol. Biol. 24*:307-331. The ALIGN program is based on the algorithm of Myers and Miller (1988) *supra*. A PAM120 weight residue table, a gap length penalty of 12, and a gap penalty of 4 can be used with the ALIGN program when comparing amino acid sequences. The BLAST programs of Altschul *et al* (1990) *J. Mol. Biol. 215*:403 are based on the algorithm of Karlin and Altschul (1990) *supra*. BLAST nucleotide searches can be performed with the BLASTN program, score = 100, wordlength = 12, to obtain nucleotide sequences homologous to a nucleotide sequence encoding a protein of the invention. BLAST protein searches can be performed with the BLASTX program, score = 50, wordlength = 3, to obtain amino acid sequences homologous to a protein or polypeptide of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST (in BLAST 2.0) can be utilized as described in Altschul *et al.* (1997) *Nucleic Acids Res. 25*:3389. Alternatively, PSI-BLAST (in BLAST 2.0) can be used to perform an iterated search that detects distant relationships between molecules. See Altschul *et al.* (1997) *supra*. When utilizing BLAST, Gapped BLAST, PSI-BLAST, the default parameters of the respective programs (e.g., BLASTN for nucleotide sequences, BLASTX for proteins) can be used. See http://www.ncbi.nlm.nih.gov. Alignment may also be performed manually by inspection.

Unless otherwise stated, sequence identity/similarity values provided herein refer to the value obtained using GAP version 10 using the following parameters: % identity using GAP Weight of 50 and Length Weight of 3; % similarity using Gap Weight of 12 and Length Weight of 4, or any equivalent program. By "equivalent program" is intended any sequence comparison program that, for any two sequences in question, generates an alignment having identical nucleotide or amino acid residue matches and an identical percent sequence identity when compared to the corresponding alignment generated by GAP Version 10.

GAP uses the algorithm of Needleman and Wunsch (1970) *J. Mol. Biol.* 48: 443-453, to find the alignment of two complete sequences that maximizes the number of matches and minimizes the number of gaps. GAP considers all possible alignments and gap positions and creates the alignment with the largest number of matched bases and the fewest gaps. It allows for the provision of a gap creation penalty and a gap extension penalty in units of matched bases. GAP must make a profit of gap creation penalty number of matches for each gap it inserts. If a gap extension penalty greater than zero is chosen, GAP must, in addition, make a profit for each gap inserted of the length of the gap times the gap extension penalty. Default gap creation penalty values and gap extension penalty values in Version 10 of the Wisconsin Genetics Software Package for protein sequences are 8 and 2, respectively. For nucleotide sequences the default gap creation penalty is 50 while the default gap extension penalty is 3. The gap creation and gap extension penalties can be expressed as an integer selected from the group of integers consisting of from 0 to 200. Thus, for example, the gap creation and gap extension penalties can be 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65 or greater.

GAP presents one member of the family of best alignments. There may be many members of this family, but no other member has a better quality. GAP displays four figures of merit for alignments: Quality, Ratio, Identity, and Similarity. The Quality is the metric maximized in order to align the sequences. Ratio is the quality divided by the number of bases in the shorter segment. Percent Identity is the percent of the symbols that actually match. Percent Similarity is the percent of the symbols that are similar. Symbols that are across from gaps are ignored. A similarity is scored when the scoring matrix value for a pair of symbols is greater than or equal to 0.50, the similarity threshold. The scoring matrix used in Version 10 of the Wisconsin Genetics Software Package is BLOSUM62 (see Henikoff and Henikoff (1989) *Proc. Natl. Acad. Sci. USA* 89:10915).

(c) As used herein, "sequence identity" or "identity" in the context of two nucleic acid or polypeptide sequences makes reference to the residues in the two sequences that are the same when aligned for maximum correspondence over a specified comparison window. When percentage of sequence identity is used in reference to proteins it is recognized that residue positions which are not identical often differ by conservative amino acid substitutions, where amino acid residues are

substituted for other amino acid residues with similar chemical properties (e.g., charge or hydrophobicity) and therefore do not change the functional properties of the molecule. When sequences differ in conservative substitutions, the percent sequence identity may be adjusted upwards to correct for the conservative nature of the substitution. Sequences that differ by such conservative substitutions are said to have "sequence similarity" or "similarity". Means for making this adjustment are well known to those of skill in the art. Typically this involves scoring a conservative substitution as a partial rather than a full mismatch, thereby increasing the percentage sequence identity. Thus, for example, where an identical amino acid is given a score of 1 and a non-conservative substitution is given a score of zero, a conservative substitution is given a score between zero and 1. The scoring of conservative substitutions is calculated, e.g., as implemented in the program PC/GENE (Intelligenetics, Mountain View, California).

(d) As used herein, "percentage of sequence identity" means the value determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison, and multiplying the result by 100 to yield the percentage of sequence identity.

(e)(i) The term "substantial identity" of polynucleotide sequences means that a polynucleotide comprises a sequence that has at least 70% sequence identity, preferably at least 80%, more preferably at least 90%, and most preferably at least 95%, compared to a reference sequence using one of the alignment programs described using standard parameters. One of skill in the art will recognize that these values can be appropriately adjusted to determine corresponding identity of proteins encoded by two nucleotide sequences by taking into account codon degeneracy, amino acid similarity, reading frame positioning, and the like. Substantial identity of amino acid sequences for these purposes normally means sequence identity of at least 60%, more preferably at least 70%, 80%, 90%, and most preferably at least 95%.

Another indication that nucleotide sequences are substantially identical is if two molecules hybridize to each other under stringent conditions. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. However, stringent conditions encompass temperatures in the range of about 1°C to about 20°C, depending upon the desired degree of stringency as otherwise qualified herein. Nucleic acids that do not hybridize to each other under stringent conditions are still substantially identical if the polypeptides they encode are substantially identical. This may occur, e.g., when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code. One indication that two nucleic acid sequences are substantially identical is when the polypeptide encoded by the first nucleic acid is immunologically cross reactive with the polypeptide encoded by the second nucleic acid.

(e)(ii) The term "substantial identity" in the context of a peptide indicates that a peptide comprises a sequence with at least 70% sequence identity to a reference sequence, preferably 80%, more preferably 85%, most preferably at least 90% or 95% sequence identity to the reference sequence over a specified comparison window. Preferably, optimal alignment is conducted using the homology alignment algorithm of Needleman and Wunsch (1970) *J. Mol. Biol. 48*:443-453. An indication that two peptide sequences are substantially identical is that one peptide is immunologically reactive with antibodies raised against the second peptide. Thus, a peptide is substantially identical to a second peptide, for example, where the two peptides differ only by a conservative substitution. Peptides that are "substantially similar" share sequences as noted above except that residue positions that are not identical may differ by conservative amino acid changes.

The use of the term "DNA constructs" herein is not intended to limit the present invention to nucleotide constructs comprising DNA. Those of ordinary skill in the art will recognize that nucleotide constructs, particularly polynucleotides and oligonucleotides, comprised of ribonucleotides and combinations of ribonucleotides and deoxyribonucleotides may also be employed in the methods disclosed herein. Thus, the DNA constructs of the present invention encompass all nucleotide constructs that can be employed in the methods of the present invention including, but not limited to, those comprised of deoxyribonucleotides, ribonucleotides, and

combinations thereof. Such deoxyribonucleotides and ribonucleotides include both naturally occurring molecules and synthetic analogues. The nucleotide constructs of the invention also encompass all forms of nucleotide constructs including, but not limited to, single-stranded forms, double-stranded forms, hairpins, stem-and-loop structures, and the like.

In the practice of embodiments of the invention, heterologous DNA can be employed. By "heterologous DNA" is intended DNA that is foreign to the genome of an organism. Such foreign DNA encompasses any DNA present in the genome of an organism that originated in the present organism or one of its progenitors by artificial methods such as, for example, transformation. Such foreign DNA also encompasses DNA native to an organism introduced into the genome of the organism *via* non-natural methods such as, for example, transformation. Related terms include "heterologous protein" and "heterologous enzyme" which are encoded by "heterologous DNA." It is recognized that such a heterologous enzyme or protein can possess an amino acid sequence that is identical to that of a native enzyme or protein of an organism. Further, a "heterologous coding sequence" is coding sequence composed of heterologous DNA.

It is recognized that enzymes similar to those described herein, referred to as "variant enzymes," may be utilized. By "variant enzyme" or "variant protein" is intended a protein derived from the native protein by deletion (so-called truncation) or addition of one or more amino acids to the N-terminal and/or C-terminal end of the native protein; deletion or addition of one or more amino acids at one or more sites in the native protein; or substitution of one or more amino acids at one or more sites in the native protein. Variant proteins encompassed by the present invention are biologically active, that is they continue to possess at least one desired biological activity of the native protein, that is, for example, 2-enoyl-CoA hydratase or 3-ketoacyl-CoA reductase activity as described herein for MFP2. Such variants may result from, for example, genetic polymorphism or from human manipulation. Biologically active variants of a native enzyme or protein of the invention will have at least about 40%, 50%, 60%, 65%, 70%, generally at least about 75%, 80%, 85%, preferably at least about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, and more preferably at least about 98%, 99% or more sequence identity to the amino acid sequence for the native protein as determined by sequence alignment programs

described elsewhere herein using default parameters. A biologically active variant of a protein of the invention may differ from that protein by as few as 1-15 amino acid residues, as few as 1-10, such as 6-10, as few as 5, as few as 4, 3, 2, or even 1 amino acid residue.

The proteins of the invention may be altered in various ways including amino acid substitutions, deletions, truncations, and insertions. Methods for such manipulations are generally known in the art. For example, amino acid sequence variants of the polypeptide can be prepared by mutations in the nucleotide sequence encoding the native protein of interest which is also referred to as the coding sequence of the native protein. Thus, proteins and their respective coding sequences include the native forms as well as variants thereof.

A variety of methods can be used to produce variant enzymes such as, for example, mutagenesis of the coding sequences of the native enzyme. Methods for mutagenesis and nucleotide sequence alterations are well known in the art. See, for example, Walker and Gaastra, eds. (1983) *Techniques in Molecular Biology* (MacMillan Publishing Company, New York); Kunkel (1985) *Proc. Natl. Acad. Sci. USA* 82:488-492; Kunkel *et al.* (1987) *Methods Enzymol.* 154:367-382; Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor, New York); U.S. Patent No. 4,873,192; and the references cited therein; herein incorporated by reference. Guidance as to appropriate amino acid substitutions that do not affect biological activity of the protein of interest may be found in the model of Dayhoff *et al.* (1978) *Atlas of Protein Sequence and Structure* (Natl. Biomed. Res. Found., Washington, D.C.), herein incorporated by reference. Conservative substitutions, such as exchanging one amino acid with another having similar properties, may be preferred.

In constructing variants of the enzyme of interest, modifications to the nucleotide sequences encoding the variants will be made such that variants continue to possess the desired activity. Obviously, any mutations made in the DNA encoding the variant protein must not place the sequence out of reading frame and preferably will not create complementary regions that could produce secondary mRNA structure. See EP Patent Application Publication No. 75,444.

In some cases it may be necessary to utilize a protein that possesses more than one enzymatic function. If only one or subset of enzymatic activities of such a protein

is desired, it will be necessary to "neutralize," that is eliminate or substantially minimize, the undesirable enzymatic activity or activities. Those skilled in the art of modifying proteins and enzymes know that a variety of methods can be used singly or in combination to neutralize an enzymatic activity. Generally such methods involve modifying the coding sequences of the protein such that the desired activity or activities are retained and the undesirable activity or activities are eliminated. Such modifications to the coding sequence include deletions, substitutions, and insertions.

The deletions, insertions, and substitutions of the protein sequences encompassed herein are not expected to produce radical changes in the characteristics of the protein. However, when it is difficult to predict the exact effect of the substitution, deletion, or insertion in advance of doing so, one skilled in the art will appreciate that the effect will be evaluated by routine screening assays. That is, the activity can be evaluated by enzyme activity assays, such as, for example, a PHA synthase assay, an enoyl-CoA hydratase assay, a ketoacyl-CoA reductase assay and an acetyl-CoA:acetyl transferase assay. See, for example, Schubert *et al.* (1988) *J. Bacteriol. 170*:5837-5847 (PHA synthase), Valentin and Steinbuechel (1994) *Appl. Microbiol. Biotechnol. 40*:699-709 (PHA synthase), Moskowitz and Merrick (1969) *Biochemistry 8*:2748-2755 (enoyl-CoA hydratase), Lynen and Wieland (1955) *Meth. Enzymol. 1*:566-573 (ketoacyl-CoA reductase), Nishimura *et al.* (1978) *Arch Microbiol. 116*:21-27 (acetyl-CoA:acetyl transferase) and Iwahashi *et al.* (1989) *J. Biochem. 105*:588-593 (NAD/NADH kinase); all of which are herein incorporated by reference.

Nucleotide sequence encoding the enzymes of the invention may be derived from a mutagenic and recombinogenic procedure such as DNA shuffling. With such a procedure, one or more different coding sequences of an enzyme of the invention can be manipulated to create a new enzyme possessing the desired properties. In this manner, libraries of recombinant polynucleotides are generated from a population of related sequence polynucleotides comprising sequence regions that have substantial sequence identity and can be homologously recombined *in vitro* or *in vivo*. For example, using this approach, nucleotide sequence motifs encoding a domain of interest in an enzyme of the invention may be shuffled to obtain a new gene coding for an enzyme with an improved or modified property of interest, such as an increased K_m or modification that results in changes in substrate or product specificities of the

enzyme. Such changes in substrate and product specificities include, but are not limited, to changes related to stereochemistry of the substrate utilized and/or the product formed. For example, an enzyme may only catalyze the formation of a (+)-epimer of a particular product. However, after shuffling one or more nucleotide sequences encoding such an enzyme, a variant enzyme is produced that produces only the (-)-epimer of the same product. Strategies for such DNA shuffling are known in the art. See, for example, Stemmer (1994) *Proc. Natl. Acad. Sci. USA 91*:10747-10751; Stemmer (1994) *Nature 370*:389-391; Crameri *et al.* (1997) *Nature Biotech. 15*:436-438; Moore *et al.* (1997) *J. Mol. Biol. 272*:336-347; Zhang *et al. (1997) Proc. Natl. Acad. Sci. USA 94*:4504-4509; Crameri *et al.* (1998) *Nature 391*:288-291; and U.S. Patent Nos. 5,605,793 and 5,837,458, herein incorporated by reference.

In embodiments of the invention, it is necessary to direct an enzyme for PHA synthesis to the peroxisomes of a plant. Methods for directing an enzyme to the peroxisome are well known in the art. Typically, such methods involve operably linking a nucleotide sequence encoding a peroxisome-targeting signal to the coding sequence of the enzyme or modifying the coding sequence of the enzyme to additionally encode the peroxisome-targeting signal without substantially affecting the intended function of the encoded enzyme. See, for example, Olsen *et al.* (1993) *Plant Cell 5*:941-952, Mullen *et al.* (1997) *Plant Physiol.* 115:881-889, Gould *et al.* (1990) *EMBO J. 9*:85-90, Flynn *et al.* (1998) *Plant J. 16*:709-720; Preisig-Muller and Kindl (1993) *Plant Mol. Biol. 22*:59-66 and Kato *et al.* (1996) *Plant Cell 8*:1601-1611; herein incorporated by reference.

It is recognized that an enzyme of the invention may be directed to the peroxisome by operably linking a peroxisome-targeting signal to the C-terminus or the N-terminus of the enzyme. It is further recognized that an enzyme which is synthesized with a peroxisome-targeting signal may be processed proteolytically *in vivo* resulting in the removal of the peroxisome-targeting signal from the amino acid sequence of the mature, peroxisome-localized enzyme.

It is recognized that it may be necessary to reduce or eliminate the activity of one or more enzymes in a plant that interfere with PHA production by the methods of the present invention. The activity of such an interfering enzyme may be reduced or eliminated by reducing or eliminating the synthesis of the interfering enzyme. Methods for reducing or eliminating the synthesis of a particular protein or enzyme in

a plant, such as, for example, sense and antisense suppression methods, are known in the art.

Antisense suppression methods involve the use of DNA construct that is portion complementary to at least a portion of a transcript encoding the protein of interest. The antisense DNA construct is designed for the production of antisense transcripts when transcribed in a plant. Such antisense transcripts are capable of hybridizing with the corresponding native or sense transcript of the protein of interest. Modifications of the antisense sequences may be made as long as the sequences hybridize to and interfere with expression of the corresponding sense transcript. In this manner, antisense constructions having 70%, preferably 80%, more preferably 85% sequence identity to the corresponding sense transcript may be used. Furthermore, portions of the antisense nucleotides may be used to disrupt the expression of the target gene. Generally, sequences of at least 50 nucleotides, 100 nucleotides, 200 nucleotides, or greater may be used.

Sense suppression methods, also known as cosuppression methods, involve the use of DNA construct that is designed to produce of a transcript that is in the same orientation, the sense orientation, as the transcript of the protein of interest. Methods for suppressing the production of a protein in a plant using nucleotide sequences in the sense orientation to are known in the art. The methods generally involve transforming plants with a DNA construct comprising a promoter that drives expression in a plant operably linked to at least a portion of a nucleotide sequence that corresponds to the transcript of the endogenous gene. Typically, such a nucleotide sequence has substantial sequence identity to the sequence of the transcript of the endogenous gene, preferably greater than about 65% sequence identity, more preferably greater than about 85% sequence identity, most preferably greater than about 95% sequence identity. See, U.S. Patent Nos. 5,283,184 and 5,034,323; herein incorporated by reference.

In the methods of the present invention, expression cassettes can be utilized. The cassette will include 5' and 3' regulatory sequences operably linked to the gene of interest. Generally, the expression cassette is provided with a plurality of restriction sites for insertion of the sequence to be under the transcriptional regulation of the regulatory regions. The expression cassette may additionally contain selectable marker genes.

By "operably linked" is intended the joining of two or more contiguous nucleotide sequences in such a manner that the desired functions or functions are achieved. "Operably linked" includes reference to a functional linkage between a promoter and a second sequence, wherein the promoter sequence initiates and mediates transcription of the DNA sequence corresponding to the second sequence. Generally, operably linked means that the nucleic acid sequences being linked are contiguous and, where necessary to join two protein coding regions, contiguous and in the same reading frame. In the case of protein coding sequences, "operably linked" includes joining two protein coding sequences in such a manner that both sequences are in the same reading frame for translation. For example, a nucleotide sequence encoding a peroxisome-targeting signal may be joined to the 3' end of a coding sequence of a protein of the invention in such manner that both sequences are in the same reading frame for translation to yield a the protein of the invention with a C-terminal addition of the peroxisome-targeting signal.

The cassette may additionally contain at least one additional gene to be cotransformed into the organism. Alternatively, the additional gene(s) can be provided on multiple expression cassettes.

The expression cassette will include in the 5'-to-3' direction of transcription, a transcriptional and translational initiation region, a nucleotide sequence of interest, and a transcriptional and translational termination region functional in plants. The transcriptional initiation region, the promoter, may be native or analogous or foreign or heterologous to the plant host. Additionally, the promoter may be the natural sequence or alternatively a synthetic sequence. By "foreign" is intended that the transcriptional initiation region is not found in the native plant into which the transcriptional initiation region is introduced.

While it may be preferable to express the sequences using heterologous promoters, the native promoter sequences may be used. Such constructs would change expression levels of MFP2 in the plant or plant cell. Thus, the phenotype of the plant or plant cell is altered.

The termination region may be native with the transcriptional initiation region, may be native with the DNA sequence of interest, or may be derived from another source. Convenient termination regions are available from the Ti-plasmid of *A. tumefaciens*, such as the octopine synthase and nopaline synthase termination regions.

See also Guerineau *et al.* (1991) *Mol. Gen. Genet. 262*:141-144; Proudfoot (1991) *Cell 64*:671-674; Sanfacon *et al.* (1991) *Genes Dev. 5*:141-149; Mogen *et al.* (1990) *Plant Cell. 2*:1261-1272; Munroe *et al.* (1990) *Gene 91*:151-158; Ballas *et al.* (1989) *Nucleic Acids Res. 17*:7891-7903; Joshi *et al.* (1987) *Nucleic Acids Res. 15*:9627-9639.

The coding sequences of the enzymes for PHA biosynthesis used in the practice of the invention can be optimized for enhanced expression in plants of interest. That is, the coding sequences can be synthesized using plant-preferred codons for improved expression. See, for example, Campbell and Gowri (1990) *Plant Physiol.* 92:1-11 for a discussion of host-preferred codon usage. Methods are available in the art for synthesizing plant-preferred genes. See, for example, U.S. Patent Nos. 5,380,831, and 5,436,391, and Murray *et al.* (1989) *Nucleic Acids Res. 17*:477-498, herein incorporated by reference. Thus, the nucleotide sequences can be optimized for expression in any plant. It is recognized that all or any part of the nucleotide sequence may be optimized or synthetic. That is, synthetic or partially optimized sequences may also be used.

Additional sequence modifications are known to enhance gene expression in a cellular host. These include elimination of sequences encoding spurious polyadenylation signals, exon-intron splice site signals, transposon-like repeats, and other such well-characterized sequences that may be deleterious to gene expression. The G-C content of the sequence may be adjusted to levels average for a given cellular host, as calculated by reference to known genes expressed in the host cell. When possible, the sequence is modified to avoid predicted hairpin secondary mRNA structures.

The expression cassettes may additionally contain 5'-leader sequences in the expression cassette construct. Such leader sequences can act to enhance translation. Translation leaders are known in the art and include: picornavirus leaders, for example, EMCV leader (Encephalomyocarditis 5'-noncoding region) (Elroy-Stein, O., Fuerst, T.R., and Moss, B. (1989) *Proc. Natl. Acad. Sci. USA 86*:6126-6130); potyvirus leaders, for example, TEV leader (Tobacco Etch Virus) (Allison *et al.* (1986); MDMV leader (Maize Dwarf Mosaic Virus); *Virology 154*:9-20), and human immunoglobulin heavy-chain binding protein (BiP), (Macejak, D.G., and P. Sarnow (1991) *Nature 353*:90-94; untranslated leader from the coat protein mRNA of alfalfa

mosaic virus (AMV RNA 4), (Jobling, S.A., and Gehrke, L., (1987) *Nature 325*:622-625; tobacco mosaic virus leader (TmV), (Gallie, D.R. *et al.* (1989) *Molecular Biology of RNA*, pages 237-256; and maize chlorotic mottle virus leader (MCMV) (Lommel, S.A. *et al.* (1991) *Virology 81*:382-385). See also, Della-Cioppa *et al.* (1987) *Plant Physiology, 84*:965-968. Other methods known to enhance translation can also be utilized, for example, introns, and the like.

In preparing the expression cassette, the various DNA fragments may be manipulated, so as to provide for the DNA sequences in the proper orientation and, as appropriate, in the proper reading frame. Toward this end, adapters or linkers may be employed to join the DNA fragments or other manipulations may be involved to provide for convenient restriction sites, removal of superfluous DNA, removal of restriction sites, or the like. For this purpose, *in vitro* mutagenesis, primer repair, restriction, annealing, re-substitutions, *e.g.*, transitions and transversions, may be involved. Such a fragment of DNA that has been manipulated by any method known to those skill in the art is referred to herein as a "DNA construct." The term "DNA construct" also encompasses expression cassettes, chimeric genes, synthetic genes, genes with modified coding sequences, and the like.

A number of promoters can be used in the practice of the invention. The promoters may be selected based on the desired timing, localization and level of expression genes encoding enzymes in a plant. Constitutive, seed-preferred, germination-preferred, tissue-preferred and chemical-regulatable promoters can be used in the practice of the invention. Such constitutive promoters include, for example, the core promoter of the Rsyn7 promoter and other constitutive promoters disclosed in WO 99/43838 and U.S. Patent No. 6,072,050; the core CaMV 35S promoter (Odell *et al.* (1985) *Nature 313*:810-812); rice actin (McElroy *et al.* (1990) *Plant Cell 2*:163-171); ubiquitin (Christensen *et al.* (1989) *Plant Mol. Biol. 12*:619-632 and Christensen *et al.* (1992) *Plant Mol. Biol. 18*:675-689); pEMU (Last *et al.* (1991) *Theor. Appl. Genet. 81*:581-588); MAS (Velten *et al.* (1984) *EMBO J. 3*:2723-2730); ALS promoter (U.S. Patent No. 5,659,026), and the like. Other constitutive promoters include, for example, U.S. Patent Nos. 5,608,149; 5,608,144; 5,604,121; 5,569,597; 5,466,785; 5,399,680; 5,268,463; and 5,608,142.

The methods of the invention are useful for producing PHA copolymers in seeds. Toward this end, the coding sequences for the enzymes of the invention may

be utilized in expression cassettes or DNA constructs with seed-preferred promoters, seed-development promoters (those promoters active during seed development), as well as seed-germination promoters (those promoters active during seed germination). Such seed-preferred promoters include, but are not limited to, Cim1 (cytokinin-induced message); cZ19B1 (maize 19 kDa zein); and celA (cellulose synthase) (see the copending application entitled "Seed-Preferred Promoters," U.S. Application Serial No. 09/377,648, filed August 19, 1999, herein incorporated by reference). Gama-zein is a preferred endosperm-specific promoter. Glob-1 is a preferred embryo-specific promoter. For dicots, particular promoters include those from the following genes: phaseolin, napin, β-conglycinin, soybean lectin, and the like. For monocots, particular promoters include those from the following genes: maize 15Kd zein, 22KD zein, 27kD zein, waxy, shrunken 1, shrunken 2, and globulin 1.

For tissue-preferred expression, the coding sequences of the invention can be operably linked to tissue-preferred promoters. For example, leaf-preferred promoters may be utilized if expression in leaves is desired. Leaf-preferred promoters are known in the art. See, for example, Yamamoto *et al.* (1997) *Plant J. 12(2)*:255-265; Kwon *et al.* (1994) *Plant Physiol. 105*:357-67; Yamamoto *et al.* (1994) *Plant Cell Physiol. 35(5)*:773-778; Gotor *et al.* (1993) *Plant J. 3*:509-18; Orozco *et al.* (1993) *Plant Mol. Biol. 23(6)*:1129-1138; and Matsuoka *et al.* (1993) *Proc. Natl. Acad. Sci. USA 90(20)*:9586-9590.

Other tissue-preferred promoters include, for example, Kawamata *et al.* (1997) *Plant Cell Physiol. 38(7)*:792-803; Hansen *et al.* (1997) *Mol Gen Genet. 254(3)*:337-343; Russell *et al.* (1997) *Transgenic Res. 6(2)*:157-168; Rinehart *et al.* (1996) *Plant Physiol. 112(3)*:1331-1341; Van Camp *et al.* (1996) *Plant Physiol. 112(2)*:525-535; Canevascini *et al.* (1996) *Plant Physiol. 112(2)*:513-524; Lam (1994) *Results Probl Cell Differ. 20*:181-196; Orozco *et al.* (1993) *Plant Mol Biol. 23(6)*:1129-1138; and Guevara-Garcia *et al.* (1993) *Plant J. 4(3)*:495-505.

In the practice of the invention, it may be desirable to use chemical-regulatable promoters to control the expression of gene in a plant. Depending upon the objective, the promoter may be a chemical-inducible promoter, where application of the chemical induces gene expression, or a chemical-repressible promoter, where application of the chemical represses gene expression. Chemical-inducible promoters are known in the art and include, but are not limited to, the maize In2-2 promoter,

which is activated by benzenesulfonamide herbicide safeners, the maize GST promoter, which is activated by hydrophobic electrophilic compounds that are used as pre-emergent herbicides, and the tobacco PR-1a promoter, which is activated by salicylic acid. Other chemical-regulatable promoters of interest include steroid-responsive promoters (see, for example, the glucocorticoid-inducible promoter in Schena *et al.* (1991) *Proc. Natl. Acad. Sci. USA 88*:10421-10425 and McNellis *et al.* (1998) *Plant J. 14(2)*:247-257) and tetracycline-inducible and tetracycline-repressible promoters (see, for example, Gatz *et al.* (1991) *Mol Gen. Genet. 227*:229-237, and U.S. Patent Nos. 5,814,618 and 5,789,156), herein incorporated by reference.

It is further recognized that the components of the expression cassette may be modified to increase expression. For example, truncated sequences, nucleotide substitutions or other modifications may be employed. See, for example Perlak *et al.*(1991) *Proc. Natl. Acad. Sci. USA* 88:3324-3328; Murray *et al.* (1989) *Nucleic Acid Research* 17:477-498; and WO 91/16432.

Transformation protocols as well as protocols for introducing nucleotide sequences into plants may vary depending on the type of plant or plant cell, i.e., monocot or dicot, targeted for transformation. Suitable methods of introducing nucleotide sequences into plant cells and subsequent insertion into the plant genome include microinjection (Crossway *et al.* (1986) *Biotechniques 4*:320-334), electroporation (Riggs *et al.* (1986) *Proc. Natl. Acad Sci. USA 83*:5602-5606, *Agrobacterium*-mediated transformation (Townsend *et al.*, U.S. Patent No. 5,563,055; Zhao *et al.*, U.S. Patent No. 5,981,840), direct gene transfer (Paszkowski *et al.* (1984) *EMBO J. 3*:2717-2722), and ballistic particle acceleration (see, for example, Sanford *et al.*, U.S. Patent No. 4,945,050; Tomes *et al.* (1995) "Direct DNA Transfer into Intact Plant Cells via Microprojectile Bombardment," in *Plant Cell, Tissue, and Organ Culture: Fundamentol Methods*, ed. Gamborg and Phillips (Springer-Verlag, Berlin); and McCabe *et al.* (1988) *Biotechnology 6*:923-926). Also see Weissinger *et al.* (1988) *Ann. Rev. Genet. 22*:421-477; Sanford *et al.* (1987) *Particulate Science and Technology 5*:27-37 (onion); Christou *et al.* (1988) *Plant Physiol. 87*:671-674 (soybean); McCabe *et al.* (1988) *Bio/Technology 6*:923-926 (soybean); Finer and McMullen (1991) *In Vitro Cell Dev. Biol.* 27P:175-182 (soybean); Singh *et al.* (1998) *Theor. Appl. Genet. 96*:319-324 (soybean); Datta *et al.* (1990) *Biotechnology 8*:736-740 (rice); Klein *et al.* (1988) *Proc. Natl. Acad. Sci. USA 85*:4305-4309

(maize); Klein *et al.* (1988) *Biotechnology 6*:559-563 (maize); Tomes, U.S. Patent No. 5,240,855; Buising *et al.*, U.S. Patent Nos. 5,322,783 and 5,324,646; Tomes *et al. (1995)* "Direct DNA Transfer into Intact Plant Cells via Microprojectile Bombardment," in *Plant Cell, Tissue, and Organ Culture: Fundamental Methods*, ed. Gamborg (Springer-Verlag, Berlin) (maize); Klein *et al.* (1988) *Plant Physiol. 91*:440-444 (maize); Fromm *et al.* (1990) *Biotechnology 8*:833-839 (maize); Hooykaas-Van Slogteren *et al.* (1984) *Nature (London) 311*:763-764; Bowen *et al.*, U.S. Patent No. 5,736,369 (cereals); Bytebier *et al.* (1987) *Proc. Natl. Acad. Sci. USA 84*:5345-5349 (Liliaceae); De Wet *et al.* (1985) in *The Experimental Manipulation of Ovule Tissues*, ed. Chapman *et al.* (Longman, New York), pp. 197-209 (pollen); Kaeppler *et al.* (1990) *Plant Cell Reports* 9:415-418 and Kaeppler *et al.* (1992) *Theor. Appl. Genet. 84*:560-566 (whisker-mediated transformation); D'Halluin *et al.* (1992) *Plant Cell 4*:1495-1505 (electroporation); Li *et al.* (1993) *Plant Cell Reports 12*:250-255 and Christou and Ford (1995) *Annals of Botany 75*:407-413 (rice); Osjoda *et al.* (1996) *Nature Biotechnology 14*:745-750 (maize via *Agrobacterium tumefaciens*); all of which are herein incorporated by reference.

The modified plant may be grown into plants in accordance with conventional ways. See, for example, McCormick *et al.* (1986) *Plant Cell. Reports 5*:81-84. These plants may then be grown, and either pollinated with the same transformed strain or different strains, and the resulting hybrid having the desired phenotypic characteristic identified. Two or more generations may be grown to ensure that the subject phenotypic characteristic is stably maintained and inherited and then seeds harvested to ensure the desired phenotype or other property has been achieved.

In the methods of the present invention, plants genetically manipulated to produce PHA are utilized. By "genetically manipulated" is intended modifying the genome of an organism, preferably a plant, including cells and tissue thereof, by any means known to those skilled in the art. Modifications to a genome include both losses and additions of genetic material as well as any sorts of rearrangements in the organization of the genome. Such modifications can be accomplished by, for example, transforming a plant's genome with a DNA construct containing nucleotide sequences which are native to the recipient plant, non-native or a combination of both, conducting a directed sexual mating or cross pollination within a single species or

between related species, fusing or transferring nuclei, inducing mutagenesis and the like.

In the practice of certain specific embodiments of the present invention, a plant is genetically manipulated to produce more than one heterologous enzyme involved in PHA synthesis. Those of ordinary skill in the art realize that this can be accomplished in any one of a number of ways. For example, each of the respective coding sequences for such enzymes can be operably linked to a promoter and then joined together in a single continuous fragment of DNA comprising a multigenic expression cassette. Such a multigenic expression cassette can be used to transform a plant to produce the desired outcome. Alternatively, separate plants can be transformed with expression cassettes containing one or a subset of the desired set of coding sequences. Transformed plants that express the desired activity can be selected by standard methods available in the art such as, for example, assaying enzyme activities, immunoblotting using antibodies which bind to the enzymes of interest, assaying for the products of a reporter or marker gene, and the like. Then, all of the desired coding sequences can be brought together into a single plant through one or more rounds of cross pollination utilizing the previously selected transformed plants as parents.

Methods for cross pollinating plants are well known to those skilled in the art, and are generally accomplished by allowing the pollen of one plant, the pollen donor, to pollinate a flower of a second plant, the pollen recipient, and then allowing the fertilized eggs in the pollinated flower to mature into seeds. Progeny containing the entire complement of heterologous coding sequences of the two parental plants can be selected from all of the progeny by standard methods available in the art as described *supra* for selecting transformed plants. If necessary, the selected progeny can be used as either the pollen donor or pollen recipient in a subsequent cross pollination.

The invention can be practiced with any plant species including, but not limited to, monocots and dicots.. Examples of plants of interest include, but are not limited to, corn or maize (*Zea mays*), *Brassica* sp. (e.g., *B. napus, B. rapa, B. juncea*), particularly those *Brassica* species useful as sources of seed oil, alfalfa (*Medicago sativa*), rice (*Oryza sativa*), rye (*Secale cereale*), sorghum (*Sorghum bicolor, Sorghum vulgare*), millet (e.g., pearl millet (*Pennisetum glaucum*), proso millet (*Panicum miliaceum*), foxtail millet (*Setaria italica*), finger millet (*Eleusine coracana*)), sunflower

(*Helianthus annuus*), safflower (*Carthamus tinctorius*), wheat (*Triticum aestivum*), soybean (*Glycine max*), tobacco (*Nicotiana tabacum*), potato (*Solanum tuberosum*), peanuts (*Arachis hypogaea*), cotton (*Gossypium barbadense*, *Gossypium hirsutum*), sweet potato (*Ipomoea batatus*), cassava (*Manihot esculenta*), coffee (*Coffea* spp.), coconut (*Cocos nucifera*), pineapple (*Ananas comosus*), citrus trees (*Citrus* spp.), cocoa (*Theobroma cacao*), tea (*Camellia sinensis*), banana (*Musa* spp.), avocado (*Persea americana*), fig (*Ficus casica*), guava (*Psidium guajava*), mango (*Mangifera indica*), olive (*Olea europaea*), papaya (*Carica papaya*), cashew (*Anacardium occidentale*), macadamia (*Macadamia integrifolia*), almond (*Prunus amygdalus*), sugar beets (*Beta vulgaris*), sugarcane (*Saccharum* spp.), oats, barley, vegetables, ornamentals, and conifers.

Preferably, plants of the present invention are crop plants (for example, maize, alfalfa, sunflower, *Brassica* sp., soybean, cotton, safflower, peanut, sorghum, wheat, rice, potatoes, millet, tobacco, etc.), more preferably maize and oilseed plants, yet more preferably maize plants. Such oilseed plants include, but are not limited to, *Brassica* sp., sunflower, safflower, soybean, peanut, cotton, flax, coconut and oil palm.

The following examples are offered by way of illustration and not by way of limitation.

EXPERIMENTAL

EXAMPLE 1: Engineering Plants to Produce PHA Copolymers

Bacteria can produce PHA copolymers because these substrates are apparently derived from the β-oxidation cycle; as bacterial cells are uncompartmented, both β-oxidation and PHA synthesis take place in the cytosol. In plants, however, β-oxidation is confined primarily to peroxisomes and thus offers a suitable site for copolymer production. Using methods known to those of ordinary skill in the art, signal sequences for targeting proteins to peroxisomes can be added to PHA-producing enzymes, allowing the localization of these enzymes in the peroxisomes. Such signal sequences for targeting proteins to plant peroxisomes are well known (Mullen *et al.* (1997) *Plant Journal 12*:313-322; Trelease *et al.* (1996) *Protoplasma 195*:156-167).

An intermediate in β-oxidation is S-(+)-3-hydroxyacyl-CoA. However, its configuration is unsuitable for PHA synthases, which require R-(-)-3-hydroxyacyl-CoA as a substrate (Gemgross *et al.* (1995) *Proc. Natl. Acad. Sci. USA 92*:6279-6283; Steinbuchel, A. (1991) *Biomaterials: Novel materials from biological materials*, D. Byrom, ed. (New York: Macmillan Publishers Ltd.), pp. 123-213). Moreover, the concentration of R-(-)-3-hydroxyacyl-CoA in the peroxisomes must be very low, as the enzyme catalyzing the proximal and distal reactions of this intermediate is multifunctional in nature (Engeland *et al.* (1991) *Eur. J. Biochem. 200*:171-178; Guhnemann Schafer *et al.* (1995) *Biochim. Biophys. Acta. 1256*:181-186). As acyl-CoA oxidase appears to be an independent enzyme (i.e., not a component of a multi-enzyme complex) in β-oxidation, its product, 2-enoyl-CoA, might be readily available for hydration into the R-(-)- epimer, provided that a hydratase capable of catalyzing this reaction is available (Figure 1).

A multifunctional protein from yeast reportedly has only the activities of enoyl-CoA hydratase and R-(-)-3-hydroxyacyl-CoA dehydrogenase (Hiltunen *et al.* (1992) *J. Biol. Chem. 267*:6646-6653). Interestingly, this enoyl-CoA hydratase forms R-(-)-3-hydroxyacyl-CoA in contrast to its plant counterpart (Guhnemann *et al.* (1994) *Eur. J. Biochem. 226*:909-915; Kindl, H. (1993) *Biochimie 75*:22R-230). Engineering the yeast multifunctional protein (encoded by GenBank Accession No. M86456, SEQ ID NO: 3) to carry only the hydratase activity and then targeting it to the plant peroxisomes along with a PHA synthase should lead to PHA copolymer formation.

Leaf *et al.* ((1996) *Microbiology 142*:1169-1180) were able to produce PHB granules in the cytosol of yeast transformed with only PHB synthase. Although they were unable to pinpoint the compartment in which it was localized, they identified a 3-hydroxybutyryl-CoA dehydrogenase that catalyzed the reversible reaction between acetoacetyl-CoA and R-(-)-3-hydroxybutyryl-CoA. No further information (i.e. gene or protein sequence) is available on this enzyme yet. Once the gene for 3-hydroxyacyl-CoA dehydrogenase enzyme becomes available, it can be used in producing substrate for PHA copolymer formation in peroxisomes. It is recognized that the coding sequence of this enzyme can be manipulated to alter characteristics of the encoded enzyme to favor the synthesis R-(-)-3-hydroxyacyl-CoA over 3-ketoacyl-CoA in plant peroxisomes by techniques well known to those skilled in the art. Such

techniques include, for example, e.g., DNA shuffling (Crameri *et al.* (1998) *Nature 391*:288-291; Stemmer, W.P.C. (1994) *Proc. Natl. Acad. Sci. USA 91*:10747-10751; Stemmer, W.P.C. (1994) *Nature 370*:389-391).

Mammalian mitochondrial enoyl-CoA hydratase is an independent enzyme (Minami-Ishi *et al.* (1989) *Eur. J. Biochem. 185*:73-78) which catalyzes the formation of S-(+)-3-hydroxyacyl-CoA (encoded by GenBank Accession No. U57486, SEQ ID NO: 22). The DNA encoding this enzyme can be subjected to shuffling to alter the activity of this enzyme to form the R-(-) instead of the S-(+)-epimer. Then, the shuffled DNA can be modified further to include nucleotide sequences which encodes a signal sequence for targeting to the peroxisomes.

Recently, a 2-enoyl-CoA hydratase from *Aeromonas caviae* was reported to hydrate 2-enoyl-CoA to R-3-hydroxyacyl-CoA (DDBJ Accession No. E15860, SEQ ID NO: 21) (Fukui *et al.* (1998) *J. Bacteriol. 180*:667-673). This enzyme can be modified to include a signal sequence for targeting to plant peroxisomes. Together with a peroxisome-localized PHA synthase, such an enoyl-CoA hydratase can catalyze the biosynthesis of copolymers.

EXAMPLE 2: Production of Specific Types of PHA in Plants

It is desirable to produce a pure copolymer of a defined monomer composition. A relatively pure copolymer would have predictable properties in comparison to a mixture of copolymers as the composition of the latter can vary according to the environment. The ability of *Pseudomonas sp.* to make copolymers of PHAs from various substrates is well known to those skilled in the art. However, the PHA synthases from these species have a broad substrate range (Caballero *et al.* (1995) *Int. J. Biol. Macromol. 17*:86-92; Huisman *et al.* (1989) *Appl. Environ. Microbiol. 55*:1949-1954; ; Lee *et al.* (1995) *Appl. Environ. Microbiol. 42*:901-909; Ramsay *et al.* (1990) *Appl. Environ. Microbiol. 56*:2093-2098; Steinbuechel *et al.* (1992) *Appl. Environ. Microbiol. 37*:691-697; Timm *et al.* (1992) *Eur. J. Biochem. 209*:1R-30). When a genomic fragment containing the PHA synthase gene from *Thiocapsa pfennigii* (see, WO 96/08566) was introduced into *Pseudomonas putida* or *A. eutrophus* strains deficient in PHA synthase, majority of the copolymer made was polyhydroxybutyrate-co-hydroxyhexanoate (HB-co-HHX) (Liebergesell *et al.* (1993)

Appl. Environ. Microbiol. 40:292-300; Valentin *et al.* (1994) *Appl. Environ. Microbiol 40*:710-716). These are the first reports of an enzyme overcoming the barrier between short- and medium-chain monomers with respect to substrate specificity for copolymer synthesis.

Recently, a PHA synthase has been identified from *A. caviae* (DDBJ Accession No. D88825, SEQ ID NO: 11) that also makes primarily poly(hydroxybutyrate-*co*-hydroxyhexanoate) copolymer when the bacteria are grown in cultures containing octanoate or (Fukui *et al.* (1997) *J. Bacteriol. 179*:4821-4830). This enzyme, when transformed into an *A. eutrophus* strain that is deficient in PHB synthase, confers upon it the ability to make poly(hydroxybutyrate-*co*-hydroxyhexanoate), indicating that this enzyme is specific for these two substrates (Fukui *et al.* (1997) *J. Bacteriol. 179*:4821-4830). Copolymers consisting mainly of poly(hydroxybutyrate-*co*-hydroxyhexanoate) can be produced if either *T. pfennigii* or *A. caviae* synthase is targeted to the plant peroxisomes along with an enoyl-CoA hydratase, 3-ketoacyl-CoA reductase, or 3-hydroxyacyl-CoA dehydrogenase that is capable of producing the R-(-) epimer (Figure 3).

By introducing the other two enzymes (ketothiolase and reductase) of the PHA biosynthetic pathway into the peroxisomes, a large portion of acetyl-CoA should be partitioned to the synthesis of PHA (Figures 1 and 3). As ketothiolase is the most limiting enzyme in β-oxidation and is not associated with other enzymes (Kindl, H. (1987) *Lipids:Structure and Function*, P.K. Stumpf, ed. (Orlando, FL: Academic Press, Inc.), pp. 31-52), 3-ketobutyryl-CoA, the penultimate product of the last cycle of β-oxidation, should be readily available to the introduced reductase for conversion into 3-hydroxybutyryl-CoA. Assuming the dominant fatty acid being degraded through β-oxidation is C_{18}, 3-ketobutyryl-CoA would constitute >20% of the carbon flux through β-oxidation. Even if the reductase can use 25% of this intermediate, that would entail a diversion of 5% carbon from fatty acids passing through β-oxidation toward PHA formation. Introducing ketothiolase would further augment the level of 3-ketobutyryl-CoA in peroxisomes.

Expression of PHB biosynthetic machinery in the peroxisomes along with that in the plastids as well as cytosol can lead to more PHB deposition in seeds. Previously, it has been reported that the expression of PHB biosynthetic enzymes in the cytosol of plants resulted in plants being of reduced vigor or "sick" (Poirier *et al.*

(1992) *Science* 256:520-523). In this study, only reductase and synthase were expressed, however, allowing the cytosolic ketothiolase to supply acetoacetyl-CoA for PHB synthesis. Acetoacetyl-CoA, however, is a substrate for the synthesis of other cellular components, such as secondary metabolites and phytohormones. Enough acetoacetyl-CoA may have been diverted toward the formation of PHB that the homeostatic limits for normal cell metabolism were diminished. Alternatively, PHB granules might have physically caused disturbance in the leaf cytosol, affecting metabolism in general. The physiology of leaves may limit their usefulness as a site of PHB synthesis. A mature leaf is a source of photosynthate and as such produces and supplies photosynthate to sinks within the plant. On the other hand, a developing seed is a strong sink. Due to the myriad physiological differences between a source leaf and a strong sink like a developing seed, expression of genes encoding enzymes involved in PHB synthesis in the cytosol of a developing seed may not be as toxic as in that of a leaf. Expression of these genes in the peroxisomes of seeds can be driven by seed-preferred, chemical-regulatable or germination-preferred promoters. If these genes need to be expressed during both seed fill and germination or during only a limited portion of the seed fill period, a chemical-regulatable promoter may be desirable.

It is further recognized that in the practice of the invention, it can be advantageous to make use of transgenic or naturally occurring lines of oilseed plants that are known to have higher rates of β-oxidation in their seeds to achieve optimal PHA production in a plant.

EXAMPLE 3: Engineering a Peroxisomal 2-Enoyl-CoA Hydratase from a Yeast Multifunctional Protein

To produce PHA in plant peroxisomes, it is essential to effectively divert 2-enoyl-CoA from β-oxidation and to the synthesis of R-(-)-3-hydroxyacyl-CoA, the substrate of PHA synthase. In contrast to the multifunctional protein in other organisms, yeast multifunctional protein (encoded by GenBank Accession No. M86456, SEQ ID NO: 3) converts *trans*-2-enoyl-CoA to R-(-)-3-hydroxyacyl-CoA. The hydratase domain of the yeast multifunctional protein utilizes a broader chain-length range of substrates than does the hydratase isolated from *Aeromonas caviae*

(Fukui *et al.* (1998) *J. Bacteriol. 180*:667-673). Such a hydratase with such a broad substrate range finds use in the production of a wide variety of copolymers in plants.

Thus, the R-specific enoyl-CoA hydratase of the yeast multifunctional protein can used to produce R-(-)-3-hydroxyacyl-CoA for PHA synthesis in plant peroxisomes. Since the R-(-)-3-hydroxyacyl-CoA dehydrogenase of the yeast multifunctional protein requires NADH and the NADH-binding sites are located in the N-terminal portion of the polypeptide, the hydratase is likely located in the C-terminal portion of the yeast multifunctional protein. Filppula *et al.* ((1995) *J. Biol. Chem. 270*:27453-27457) constructed a C-terminally truncated form of the yeast multifunctional protein and showed that the mutant enzyme contained only R-(-)-3-hydroxyacyl-CoA dehydrogenase activity and thus demonstrated that the deleted C-terminal portion of the full-length yeast multifunctional protein is essential for hydratase activity. Qin *et al.* (1997) *Biochem. J. 321*: 21-28 described an N-truncated rat multifunctional protein with 318 amino acid residue deletion. This mutant enzyme remained full hydratase activity while its dehydrogenase activity was completely lost. These observations are in agreement with the prediction that an N-terminally truncated form of yeast multifunctional protein possess hydratase activity and lack dehydrogenase activity.

To engineer a 2-enoyl-CoA hydratase that catalyzes the synthesis of R-(-)-hydroxyacyl-CoA, the nucleotide sequence encoding the yeast multifunctional protein (GenBank Accession No. M86456, SEQ ID NO: 3) can be modified by site-directed mutagenesis and/or N-terminal truncation to remove the dehydrogenase activity. After analyzing the primary structure of the yeast multifunctional protein, two putative NADH-binding domains (residues 152-180 and residues 456-484) were identified. These two putative NADH-binding domains each possess the conserved Y and K that are a signature NADH-binding-sites. Y165 and K169 lie in the first domain while Y478 and K482 are found in the second domain, although it is unknown whether the first domain, the second one or both of them serves for NAD/NADH-binding. To eliminate dehydrogenase activity, the NADH binding sites can be disrupted to generate mutant yeast multifunctional proteins such as, for example, a first mutant enzyme having the two amino acid substitutions, Y165F and K169A and a second mutant enzyme having the two amino acid substitutions Y478F and K482A and a third mutant enzyme having the four amino acid substitutions,

Y165F, K169A, Y478F, and K482A. Alternatively, two N-terminally truncated versions of the yeast multifunctional protein can be constructed by eliminating one and both NADH-binding sites (Figure 2). Methods for assaying such enzyme activities are known in the art. See, for example, Moskowitz and Merrick (1969) *Biochemistry 8*:2748-2755 (enoyl-CoA hydratase), and Lynen and Wieland (1955) *Meth. Enzymol. 1*:566-573 (ketoacyl-CoA reductase) and Example 6 infra.

An N-terminally truncated version of the yeast multifunctional protein is set forth in SEQ ID NO: 5. A nucleotide sequence for the truncated yeast multifunctional protein is set forth in SEQ ID NO: 4. A similar approach can be used to modify any multifunctional protein known in the art.

The resulting 2-enoyl-CoA hydratase enzyme can then be modified for targeting to the peroxisome by operably linking a peroxisome-targeting signal sequence to the coding sequence for the mutant enzyme.

EXAMPLE 4: Engineering a Peroxisomal 3-ketoacyl-CoA Reductase from a Yeast Multifunctional Protein

R-(-)-3-hydroxybutyryl-coenzyme A dehydrogenase (also known as acetoacetyl-CoA reductase) is encoded by *phaB* in PHA biosynthesis in a number of microorganisms. Such R-(-)-3-hydroxybutyryl-coenzyme A dehydrogenases all utilize NADPH as the electron donor. In one report an acetoacetyl-CoA reductase was shown to be NADH-dependent, but it produced S-(+)-3-hydroxybutyryl-CoA as its product (Liebergesell and Steinbuchel (1992) *Eur. J. Biochem. 209*:135-150). Another report showed that an NADH-dependent acetoacetyl-CoA reductase was isolated from *Parracoccus denitrificans* (Yabutani (1995) *FEMS Microbiol. Lett. 133*:85-90). Subsequent characterization confirmed that it utilized NADPH, but not NADH as an electron donor (Madison and Huisman (1999) *Microbiol. Mol. Biol. Rev. 63*:21-53).

In plant peroxisomes, it is postulated that NADPH pool is limited while NADH predominates. It is doubtful that the *phaB* gene product, an NADPH-dependent reductase, will function in peroxisomes. Therefore, a new enzyme that utilizes NADH as the electron donor is desired. The desired enzyme must also be able to convert 3-acetoacetyl-CoA and to R-(-)-3-hydroxybutyryl-CoA. Preferably,

the desired enzyme is also capably of converting any 3-ketoacyl-CoA to an R-(-)-3-hydroxyacyl-CoA.

Using protein engineering methods, the coding sequence of the yeast multifunctional protein can be modified to produce the desired enzyme. The dehydrogenase moiety of the yeast multifunctional protein utilizes R-(-)-3-hydroxyacyl-CoA and requires NADH as its electron acceptor. Since the dehydrogenase of the yeast multifunctional protein enzyme utilizes substrates of fatty acyl-CoAs with C_4 or longer chain length, the desired enzyme is expected to catalyze the NADH-dependent reduction of acetoacetyl-CoA to R-(-)-3-hydroxybutyryl-CoA. Based on sequence analysis of primary structure of the protein, two NADH-binding sites residing at the N-terminal portion were identified. For example, to produce the desired 3-ketoacyl-CoA reductase, the nucleotide sequence encoding the of yeast multifunctional protein can be truncated to produce a nucleotide sequence that encodes a C-terminally truncated version of the yeast multifunctional protein which lacks the final 271 amino acid of amino acid sequence of the yeast multifunctional protein. The C-terminally truncated version of the yeast multifunctional protein is set forth in SEQ ID NO: 7. A nucleotide sequence encoding the truncated yeast multifunctional protein is set forth in SEQ ID NO: 6.

The desired 3-ketoacyl-CoA reductase can then be modified, if necessary, for targeting to the peroxisome by operably linking a peroxisome-targeting signal sequence to the coding sequence for the desired enzyme.

EXAMPLE 5: A Novel Maize Protein with Homology to Yeast MFP2

To search for homologous sequences in maize, the nucleotide sequence encoding the yeast MFP2 (GenBank Accession No. M86456, SEQ ID NO: 3) was used to search a Pioneer Hi-Bred maize EST database. An EST clone with substantial homology to the yeast sequence was identified. The 1362 bp maize EST clone was sequenced and found to be full-length (SEQ ID NO: 1) with an open reading frame encoding a polypeptide of 314 amino acids (SEQ ID NO: 2). Interestingly, in comparison, the yeast MFP2 is comprised of an amino acid sequence which is 900 amino acids in length. Further sequence analysis revealed that the maize amino acid sequence corresponds to the C-terminal portion of the yeast MFP2 that is believed to

be a 2-enoyl-CoA hydratase domain (Figure 2) Unlike the yeast MFP2, the smaller maize MFP2-like polypeptide lacks the dehydrogenase domain of the yeast and mammalian MFP2s and does not appear to contain a peroxisome-targeting sequence.

Thus, on the basis of homology to the yeast MFP2, the maize MFP2-like polypeptide is predicted to encode a 2-enoyl-CoA hydratase. Such a hydratase can be targeted to the peroxisome for use in PHA production therein by operably linking a peroxisome-targeting signal sequence to the coding sequence for the maize polypeptide.

The present invention discloses a novel protein from maize with homology to MFP2s. While MFP2s are found in several fungi and in several mammals including, but not limited to, mouse, guinea pig, human and pig, no plant sequence has yet been publically disclosed. It has been generally believed that MFPs are unique to fungi and animals. The discovery of the maize MFP2-like polypeptide, which shares substantial homology with the yeast MFP2, indicates, however that, at least, the hydratase domain of MFP2 is also found in plants.

While the maize MFP2-like polypeptide shares significant homology to MFP2s from fungi and mammals, the maize protein possesses several notable differences. In addition to the lack of a dehydrogenase domain, the maize MFP2-like polypeptide does not contains any known peroxisomal-targeting sequences in its N-terminal or C-terminal portions. This is different from yeast and mammalian MFP2s which are considered to be localized in peroxisomes and play roles in fatty acid β-oxidation. Thus, the maize MFP2-like polypeptide is likely to play a physiological role in plants that is distinct from that of known MFP2s.

EXAMPLE 6: Assaying the Activities of PHA Synthesis Enzymes

To test the activity of the maize MFP2-like polypeptide and the truncated yeast MFP2 (hydratase domain) prepared as described *supra*, their respective nucleotide sequences (SEQ ID NOs: 1 and 4) and that of the full-length yeast MFP2 (SEQ ID NO: 3) were cloned into appropriate bacterial expression vectors and used to transform *E. coli* using standard techniques known in the art for expressing recombinant proteins and transforming bacteria. Total protein extracts were isolated from IPTG-induced *E. coli* (strain BL21) harboring a plasmid comprising the

nucleotide sequence of SEQ ID NO: 3 (pYMFP), the nucleotide sequence of SEQ ID NO: 4 (pYHL) or the nucleotide sequence of SEQ ID NO: 1 (pMHL). The activities of the truncated proteins were tested using the functional assay schematically illustrated in Figure 4. The reaction mixture consisted of 20 µl of PHA synthase preparation (19mg/ml), 50 µl of *E. coli* extract, 50 mM Tris-HCl, pH 7.5 and µM of crotonyl-CoA, acetoacetyl-CoA (AcAcCoA), or 3-hydroxybutryl-CoA (3HB-CoA) in total volume of 300 µl. The reaction was started by the addition of the substrate and incubated at 37°C for two hours.

The results are presented in Table 1 and Figure 5. With crotonyl-CoA as a substrate, polymer formation was detected in cultures of *E .coli* that were transformed with a nucleotide sequences encoding either the full-length yeast MFP2 (pYMFP), the truncated yeast MFP2 (yYHL), or the maize MFP2-like polypeptide (yMHL). The vector-only control (Table 1, reaction 1) indicated that polymer was not formed in the presence of crotonyl-CoA without one of the three nucleotide sequences (SEQ ID NOs: 1, 3, and 4). Thus, proteins encoded by each of the nucleotide sequences can catalyze the conversion of a crotonyl-CoA, which is a 2-enoyl-CoA, into R-(-)-3-hydroxyacyl-CoA, which can then be used for PHA synthesis as a substatrate for PHA synthase.

Comparison of the relative hydratase activities, as measured by PHA formation, for the proteins encoded by SEQ ID NOs: 1, 3 and 4 is provided in Figure 5. The highest relative hydratase activity was detected in the cultures expressing the maize MFP2-like polypeptide (maize hydratase), followed by the truncated yeast MFP2 (yeast hydratase) and the full-length yeast MFP2 (yeast MFP). The results indicate that the removal an N-terminal portion of an MFP2 can lead to increased hydratase activity, as determined by increased PHA production, when compared to the hydratase activity of the full-length MFP2.

In general, the results presented in Table 1 and Figure 5 demonstrate that both the maize MFP2-like polypeptide and the truncated yeast MFP2 possess a hydratase activity that can be used for PHA synthesis in a host cell. Given that 2-enoyl-CoA substrates are found in the peroxisome, the maize MFP2-like polypeptide and the truncated yeast MFP2 of the invention can be targeted to peroxisome, along with other necessary enzymes, for PHA synthesis therein. The results further demonstrate

that a truncated MFP2 can provide an improvement in PHA production in an living organism, when compared to the full-length MFP.

Table 1

Functional Assay for the Presence of an 2-enoyl-CoA Hydratase Activity

Reaction	Substrate	Hydratase	Polymer Formed
1	crotonyl-CoA	vector only	No
2	crotonyl-CoA	pMHL	Yes
3	crotonyl-CoA	pYHL	Yes
4	crotonyl-CoA	pYMFP	Yes
5	3HB-CoA	50 µl buffer	Yes
6	AcAcCoA	50 µl buffer	No
7	AcAcCoA	pMHL	No

EXAMPLE 7: Transformation and Regeneration of Transgenic Maize Plants by Particle Bombardment

Immature maize embryos from greenhouse donor plants are bombarded with a plasmid comprising a nucleotide sequence of the invention encoding an enzyme involved in PHA synthesis operably linked to a seed-preferred promoter and the selectable marker gene PAT (Wohlleben *et al.* (1988) *Gene* 70:25-37), which confers resistance to the herbicide Bialaphos. Alternatively, the selectable marker gene is provided on a separate plasmid. Transformation is performed as follows. Media recipes follow below.

Preparation of Target Tissue

The ears are husked and surface sterilized in 30% Clorox bleach plus 0.5% Micro detergent for 20 minutes, and rinsed two times with sterile water. The immature embryos are excised and placed embryo axis side down (scutellum side up),

25 embryos per plate, on 560Y medium for 4 hours and then aligned within the 2.5-cm target zone in preparation for bombardment.

Preparation of DNA

A plasmid vector comprising the nucleotide sequence of the invention encoding an enzyme involved in PHA synthesis linked to a seed-preferred promoter is made. This plasmid DNA plus plasmid DNA containing a PAT selectable marker is precipitated onto 1.1 µm (average diameter) tungsten pellets using a $CaCl_2$ precipitation procedure as follows:

100 µl prepared tungsten particles in water
10 µl (1 µg) DNA in Tris EDTA buffer (1 µg total DNA)
100 µl 2.5 M $CaCl_2$
10 µl 0.1 M spermidine

Each reagent is added sequentially to the tungsten particle suspension, while maintained on the multitube vortexer. The final mixture is sonicated briefly and allowed to incubate under constant vortexing for 10 minutes. After the precipitation period, the tubes are centrifuged briefly, liquid removed, washed with 500 ml 100% ethanol, and centrifuged for 30 seconds. Again the liquid is removed, and 105 µl 100% ethanol is added to the final tungsten particle pellet. For particle gun bombardment, the tungsten/DNA particles are briefly sonicated and 10 µl spotted onto the center of each macrocarrier and allowed to dry about 2 minutes before bombardment.

Particle Gun Treatment

The sample plates are bombarded at level #4 in particle gun #HE34-1 or #HE34-2. All samples receive a single shot at 650 PSI, with a total of ten aliquots taken from each tube of prepared particles/DNA.

Subsequent Treatment

Following bombardment, the embryos are kept on 560Y medium for 2 days, then transferred to 560R selection medium containing 3 mg/liter Bialaphos, and subcultured every 2 weeks. After approximately 10 weeks of selection, selection-resistant callus clones are transferred to 288J medium to initiate plant regeneration. Following somatic embryo maturation (2-4 weeks), well-developed somatic embryos are transferred to medium for germination and transferred to the lighted culture room.

Approximately 7-10 days later, developing plantlets are transferred to 272V hormone-free medium in tubes for 7-10 days until plantlets are well established. Plants are then transferred to inserts in flats (equivalent to 2.5" pot) containing potting soil and grown for 1 week in a growth chamber, subsequently grown an additional 1-2 weeks in the greenhouse, then transferred to classic 600 pots (1.6 gallon) and grown to maturity. Plants are monitored and scored for PHA content and/or the activity or level of the enzyme of the invention.

Bombardment and Culture Media

Bombardment medium (560Y) comprises 4.0 g/l N6 basal salts (SIGMA C-1416), 1.0 ml/l Eriksson's Vitamin Mix (1000X SIGMA-1511), 0.5 mg/l thiamine HCl, 120.0 g/l sucrose, 1.0 mg/l 2,4-D, and 2.88 g/l L-proline (brought to volume with D-I H_20 following adjustment to pH 5.8 with KOH); 2.0 g/l Gelrite (added after bringing to volume with D-I H_20); and 8.5 mg/l silver nitrate (added after sterilizing the medium and cooling to room temperature). Selection medium (560R) comprises 4.0 g/l N6 basal salts (SIGMA C-1416), 1.0 ml/l Eriksson's Vitamin Mix (1000X SIGMA-1511), 0.5 mg/l thiamine HCl, 30.0 g/l sucrose, and 2.0 mg/l 2,4-D (brought to volume with D-I H_20 following adjustment to pH 5.8 with KOH); 3.0 g/l Gelrite (added after bringing to volume with D-I H_20); and 0.85 mg/l silver nitrate and 3.0 mg/l bialaphos(both added after sterilizing the medium and cooling to room temperature).

Plant regeneration medium (288J) comprises 4.3 g/l MS salts (GIBCO 11117-074), 5.0 ml/l MS vitamins stock solution (0.100 g nicotinic acid, 0.02 g/l thiamine HCL, 0.10 g/l pyridoxine HCL, and 0.40 g/l glycine brought to volume with polished D-I H_20) (Murashige and Skoog (1962) *Physiol. Plant. 15*:473), 100 mg/l myo-inositol, 0.5 mg/l zeatin, 60 g/l sucrose, and 1.0 ml/l of 0.1 mM abscisic acid (brought to volume with polished D-I H_20 after adjusting to pH 5.6); 3.0 g/l Gelrite (added after bringing to volume with D-I H_20); and 1.0 mg/l indolcacetic acid and 3.0 mg/l bialaphos (added after sterilizing the medium and cooling to 60°C). Hormone-free medium (272V) comprises 4.3 g/l MS salts (GIBCO 11117-074), 5.0 ml/l MS vitamins stock solution (0.100 g/l nicotinic acid, 0.02 g/l thiamine HCL, 0.10 g/l pyridoxine HCL, and 0.40 g/l glycine brought to volume with polished D-I H_20), 0.1 g/l myo-inositol, and 40.0 g/l sucrose (brought to volume with polished D-I H_20 after

adjusting pH to 5.6); and 6 g/l bacto-agar (added after bringing to volume with polished D-I H_2O), sterilized and cooled to 60° C.

EXAMPLE 8: Production of Transgenic Maize Plants via *Agrobacterium*-Mediated Transformation

For *Agrobacterium*-mediated transformation of maize with a nucleotide sequence of the invention encoding an enzyme involved in PHA synthesis, preferably the method of Zhao is employed (U.S. Patent No. 5,981,840, and PCT patent publication WO98/32326, the contents of which are hereby incorporated by reference). Briefly, immature embryos are isolated from maize and the embryos contacted with a suspension of *Agrobacterium*, where the bacteria are capable of transferring the nucleotide sequence of the invention to at least one cell of at least one of the immature embryos (step 1: the infection step). In this step the immature embryos are preferably immersed in an *Agrobacterium* suspension for the initiation of inoculation. The embryos are co-cultured for a time with the *Agrobacterium* (step 2: the co-cultivation step). Preferably the immature embryos are cultured on solid medium following the infection step. Following this co-cultivation period an optional "resting" step is contemplated. In this resting step, the embryos are incubated in the presence of at least one antibiotic known to inhibit the growth of *Agrobacterium* without the addition of a selective agent for plant transformants (step 3: resting step). Preferably the immature embryos are cultured on solid medium with antibiotic, but without a selecting agent, for elimination of *Agrobacterium* and for a resting phase for the infected cells. Next, inoculated embryos are cultured on medium containing a selective agent and growing transformed callus is recovered (step 4: the selection step). Preferably, the immature embryos are cultured on solid medium with a selective agent resulting in the selective growth of transformed cells. The callus is then regenerated into plants (step 5: the regeneration step), and preferably calli grown on selective medium are cultured on solid medium to regenerate the plants.

EXAMPLE 9: Production of Transformed Soybean Plants

Soybean embryos are bombarded with a plasmid comprising a nucleotide sequence of the invention encoding an enzyme involved in PHA synthesis operably linked to a seed-preferred as follows. To induce somatic embryos, cotyledons, 3-5 mm in length dissected from surface-sterilized, immature seeds of the soybean cultivar A2872, are cultured in the light or dark at 26°C on an appropriate agar medium for six to ten weeks. Somatic embryos producing secondary embryos are then excised and placed into a suitable liquid medium. After repeated selection for clusters of somatic embryos that multiplied as early, globular-staged embryos, the suspensions are maintained as described below.

Soybean embryogenic suspension cultures can maintained in 35 ml liquid media on a rotary shaker, 150 rpm, at 26°C with florescent lights on a 16:8 hour day/night schedule. Cultures are subcultured every two weeks by inoculating approximately 35 mg of tissue into 35 ml of liquid medium.

Soybean embryogenic suspension cultures may then be transformed by the method of particle gun bombardment (Klein *et al.* (1987) *Nature* (London) *327*:70-73, U.S. Patent No. 4,945,050). A Du Pont Biolistic PDS1000/HE instrument (helium retrofit) can be used for these transformations.

A selectable marker gene that can be used to facilitate soybean transformation is a transgene composed of the 35S promoter from Cauliflower Mosaic Virus (Odell *et al.* (1985) *Nature 313*:810-812), the hygromycin phosphotransferase gene from plasmid pJR225 (from *E. coli*; Gritz *et al.* (1983) *Gene 25*:179-188), and the 3' region of the nopaline synthase gene from the T-DNA of the Ti plasmid of *Agrobacterium tumefaciens*. The expression cassette comprising the nucleotide sequence of the invention operably linked to the seed-preferred promoter can be isolated as a restriction fragment. This fragment can then be inserted into a unique restriction site of the vector carrying the marker gene.

To 50 µl of a 60 mg/ml 1 µm gold particle suspension is added (in order): 5 µl DNA (1 µg/µl), 20 µl spermidine (0.1 M), and 50 µl $CaCl_2$ (2.5 M). The particle preparation is then agitated for three minutes, spun in a microfuge for 10 seconds and

the supernatant removed. The DNA-coated particles are then washed once in 400 µl 70% ethanol and resuspended in 40 µl of anhydrous ethanol. The DNA/particle suspension can be sonicated three times for one second each. Five microliters of the DNA-coated gold particles are then loaded on each macro carrier disk.

Approximately 300-400 mg of a two-week-old suspension culture is placed in an empty 60x15 mm petri dish and the residual liquid removed from the tissue with a pipette. For each transformation experiment, approximately 5-10 plates of tissue are normally bombarded. Membrane rupture pressure is set at 1100 psi, and the chamber is evacuated to a vacuum of 28 inches mercury. The tissue is placed approximately 3.5 inches away from the retaining screen and bombarded three times. Following bombardment, the tissue can be divided in half and placed back into liquid and cultured as described above.

Five to seven days post bombardment, the liquid media may be exchanged with fresh media, and eleven to twelve days post-bombardment with fresh media containing 50 mg/ml hygromycin. This selective media can be refreshed weekly. Seven to eight weeks post-bombardment, green, transformed tissue may be observed growing from untransformed, necrotic embryogenic clusters. Isolated green tissue is removed and inoculated into individual flasks to generate new, clonally propagated, transformed embryogenic suspension cultures. Each new line may be treated as an independent transformation event. These suspensions can then be subcultured and maintained as clusters of immature embryos or regenerated into whole plants by maturation and germination of individual somatic embryos.

EXAMPLE 10: Genetic Transformation of Sunflower Plants

Sunflower meristem tissues are transformed with an expression cassette comprising a nucleotide sequence of the invention encoding an enzyme involved in PHA synthesis operably linked to a seed-preferred promoter as follows (see also European Patent Number EP 0 486233, herein incorporated by reference, and Malone-Schoneberg *et al.* (1994) *Plant Science 103*:199-207). Mature sunflower seed (*Helianthus annuus* L.) are dehulled using a single wheat-head thresher. Seeds are surface sterilized for 30 minutes in a 20% Clorox bleach solution with the addition of

two drops of Tween 20 per 50 ml of solution. The seeds are rinsed twice with sterile distilled water.

Split embryonic axis explants are prepared by a modification of procedures described by Schrammeijer *et al.* (Schrammeijer *et al.*(1990) *Plant Cell Rep. 9*: 55-60). Seeds are imbibed in distilled water for 60 minutes following the surface sterilization procedure. The cotyledons of each seed are then broken off, producing a clean fracture at the plane of the embryonic axis. Following excision of the root tip, the explants are bisected longitudinally between the primordial leaves. The two halves are placed, cut surface up, on GBA medium consisting of Murashige and Skoog mineral elements (Murashige *et al.* (1962) *Physiol. Plant. 15*: 473-497), Shepard's vitamin additions (Shepard (1980) in *Emergent Techniques for the Genetic Improvement of Crops* (University of Minnesota Press, St. Paul, Minnesota), 40 mg/l adenine sulfate, 30 g/l sucrose, 0.5 mg/l 6-benzyl-aminopurine (BAP), 0.25 mg/l indole-3-acetic acid (IAA), 0.1 mg/l gibberellic acid (GA_3), pH 5.6, and 8 g/l Phytagar.

The explants are subjected to microprojectile bombardment prior to *Agrobacterium* treatment (Bidney *et al.* (1992) *Plant Mol. Biol. 18*: 301-313). Thirty to forty explants are placed in a circle at the center of a 60 X 20 mm plate for this treatment. Approximately 4.7 mg of 1.8 mm tungsten microprojectiles are resuspended in 25 ml of sterile TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 8.0) and 1.5 ml aliquots are used per bombardment. Each plate is bombarded twice through a 150 mm nytex screen placed 2 cm above the samples in a PDS 1000® particle acceleration device.

Disarmed *Agrobacterium tumefaciens* strain EHA105 is used in all transformation experiments. A binary plasmid vector comprising the expression cassette that contains the nucleotide sequence of the invention encoding an enzyme involved in PHA synthesis operably linked to the seed-preferred promoter is introduced into *Agrobacterium* strain EHA105 via freeze-thawing as described by Holsters *et al.* (1978) *Mol. Gen. Genet. 163*:181-187. This plasmid further comprises a kanamycin selectable marker gene (i.e. *nptII*). Bacteria for plant transformation experiments are grown overnight (28°C and 100 RPM continuous agitation) in liquid YEP medium (10 gm/l yeast extract, 10 gm/l Bactopeptone, and 5 gm/l NaCl, pH 7.0) with the appropriate antibiotics required for bacterial strain and binary plasmid

maintenance. The suspension is used when it reaches an OD_{600} of about 0.4 to 0.8. The *Agrobacterium* cells are pelleted and resuspended at a final OD_{600} of 0.5 in an inoculation medium comprised of 12.5 mM MES pH 5.7, 1 gm/l NH_4Cl, and 0.3 gm/l $MgSO_4$.

Freshly bombarded explants are placed in an *Agrobacterium* suspension, mixed, and left undisturbed for 30 minutes. The explants are then transferred to GBA medium and co-cultivated, cut surface down, at 26°C and 18-hour days. After three days of co-cultivation, the explants are transferred to 374B (GBA medium lacking growth regulators and a reduced sucrose level of 1%) supplemented with 250 mg/l cefotaxime and 50 mg/l kanamycin sulfate. The explants are cultured for two to five weeks on selection and then transferred to fresh 374B medium lacking kanamycin for one to two weeks of continued development. Explants with differentiating, antibiotic-resistant areas of growth that have not produced shoots suitable for excision are transferred to GBA medium containing 250 mg/l cefotaxime for a second 3-day phytohormone treatment. Leaf samples from green, kanamycin-resistant shoots are assayed for the presence of NPTII by ELISA and for the presence of transgene expression by assaying, for example, for PHA production as described *supra*.

NPTII-positive shoots are grafted to Pioneer® hybrid 6440 *in vitro*-grown sunflower seedling rootstock. Surface sterilized seeds are germinated in 48-0 medium (half-strength Murashige and Skoog salts, 0.5% sucrose, 0.3% gelrite, pH 5.6) and grown under conditions described for explant culture. The upper portion of the seedling is removed, a 1 cm vertical slice is made in the hypocotyl, and the transformed shoot inserted into the cut. The entire area is wrapped with parafilm to secure the shoot. Grafted plants can be transferred to soil following one week of *in vitro* culture. Grafts in soil are maintained under high humidity conditions followed by a slow acclimatization to the greenhouse environment. Transformed sectors of T_0 plants (parental generation) maturing in the greenhouse are identified by NPTII ELISA and/or by analysis in leaf extracts of enzyme activity of the enzyme encoded by the nucleotide sequence of the invention of leaf extracts, while transgenic seeds harvested from NPTII-positive T_0 plants are identified by similar enzyme activity analyses of small portions of dry seed cotyledons.

All publications and patent applications mentioned in the specification are indicative of the level of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

THAT WHICH IS CLAIMED:

1. An isolated nucleotide molecule comprising a nucleotide sequence selected from the group consisting of:

(a) the nucleotide sequence set forth in SEQ ID NO: 1;

(b) a nucleotide sequence encoding the amino acid sequence set forth in SEQ ID NO: 2;

(c) a nucleotide sequence having at least 50% sequence identity to the nucleotide sequence set forth in SEQ ID NO: 1;

(d) an antisense nucleotide sequence corresponding to the nucleotide sequence of (a), (b) or (c); and

(e) a nucleotide sequence that hybridizes under stringent conditions to the nucleotide sequence of (a).

2. An expression cassette comprising a nucleotide sequence of a nucleotide molecule of claim 1, said nucleotide sequence operably linked to a promoter that drives expression in a host cell.

3. A host cell transformed with the expression cassette of claim 2.

4. The host cell of claim 3, wherein said host cell is selected from the group consisting of a plant cell, a bacterial cell, and a yeast cell.

5. An isolated polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 2, or an amino acid sequence having at least 60% sequence identity to the amino acid sequence set forth in SEQ ID NO: 2.

6. A method for producing an enzyme for polyhydroxyalkanoate synthesis comprising:

(a) providing a coding sequence for a multifunctional protein-2;

(b) producing a modified coding sequence from said coding sequence, wherein said modified coding sequence encodes a polypeptide

comprising a 3-ketoacyl-CoA reductase or a 2-enoyl-CoA hydratase; and

(c) transforming a host cell or plant with said modified coding sequence so as to produce said enzyme in said cell or said plant.

7. The method of claim 6, wherein said coding sequence comprises at least a portion of a nucleotide sequence set forth in SEQ ID NO: 3 or SEQ ID NO: 22.

8. The method of claim 6, wherein said modified coding sequence comprises the nucleotide sequence set forth in SEQ ID NO: 4 or 6.

9. The method of claim 6 further comprising before transformation, operably linking a promoter that drives expression in a plant cell to said modified coding sequence.

10. The method of claim 9 further comprising before transformation, operably linking a nucleotide sequence encoding a peroxisome-targeting signal to said modified coding sequence.

11. The method of claim 6, wherein at least one of a start codon and a stop codon was operably linked to said modified coding sequence.

12. A modified coding sequence produced by the method of claim 6.

13. A tranformed host cell produced by the method of claim 6.

14. A transformed plant produced by the method of claim 6.

15. A transgenic plant comprising in its genome at least one stably integrated DNA construct comprising a nucleotide sequence operably linked to a promoter that drives expression in a plant, wherein said nucleotide sequence is selected from the group consisting of:

(a) the nucleotide sequence set forth in SEQ ID NO: 1;

(b) a nucleotide sequence encoding the amino acid sequence set forth in SEQ ID NO: 2;

(c) a nucleotide sequence having at least 50% sequence identity to the nucleotide sequence set forth in SEQ ID NO: 1;

(d) an antisense nucleotide sequence corresponding to the nucleotide sequence of (a), (b) or (c); and

(e) a nucleotide sequence that hybridizes under stringent conditions to the nucleotide sequence of (a).

16. The plant of claim 15, wherein said promoters are selected from the group consisting of seed-preferred promoters, chemical-regulatable promoters, germination-preferred promoters, and leaf-preferred promoters.

17. The plant of claim 15, wherein said plant is a monocot.

18. The plant of claim 17, wherein said monocot is selected from the group consisting of maize, wheat, rice, sorghum, rye, millet, barley, palm and banana.

19. The plant of claim 15, wherein said plant is a dicot.

20. The plant of claim 19, wherein said dicot is selected from the group consisting of soybean, sunflower, safflower, alfalfa, potato, *Brassica* spp., cotton, tomato, tobacco and peanut.

21. A plant genetically manipulated to produce polyhydroxyalkanoate in its peroxisomes, said plant comprising in its genome:

a stably integrated first DNA construct comprising a promoter that drives expression in a plant cell operably linked to a first coding sequence, wherein said first coding sequence encodes a polyhydroxyalkanoate synthase and is operably linked to a nucleotide sequence encoding a peroxisome-targeting signal; and

a stably integrated second DNA construct comprising a promoter that drives expression in a plant cell operably linked to a second coding sequence, wherein said second coding sequence is operably linked to a nucleotide sequence encoding a

peroxisome-targeting signal and said second coding sequence is selected from the group consisting of:

(a) a nucleotide sequence encoding a 2-enoyl-CoA hydratase that is capable of catalyzing the synthesis of R-(-)-3-hydroxyacyl-CoA ;

(b) a nucleotide sequence set forth in SEQ ID NO: 21;

(c) a nucleotide sequence comprising the 2-enoyl-CoA hydratase domain of a multifunctional protein-2.

(d) a nucleotide sequence set forth in SEQ ID NO: 4;

(e) a nucleotide sequence set forth in SEQ ID NO: 1; and

(f) a nucleotide sequence encoding a multifunctional protein-2, wherein the dehydrogenase activity of said multifunctional protein has been eliminated.

22. The plant of claim 21, wherein said promoters are selected from the group consisting of seed-preferred promoters, chemical-regulatable promoters, germination-preferred promoters, and leaf-preferred promoters.

23. The plant of claim 21, wherein said polyhydroxyalkanoate synthase is capable of catalyzing the synthesis of polyhydroxyalkanoate copolymers.

24. The plant of claim 23, wherein said polyhydroxyalkanoate synthase is encoded by a nucleotide sequence selected from the group consisting SEQ ID NOs: 8-12.

25. The plant of claim 21 further comprising in its genome a stably integrated third DNA construct comprising a promoter that drives expression in a plant cell operably linked to a third coding sequence, wherein said third coding sequence encodes a 3-ketoacyl-CoA reductase and is operably linked to a nucleotide sequence encoding a peroxisome-targeting signal.

26. The plant of claim 21, wherein said third coding sequence comprises at least a portion of a nucleotide sequence selected from the group consisting of:

(a) a nucleotide sequence encoding a 3-ketoacyl-CoA reductase that is capable of utilizing NADH;

(b) a nucleotide sequence set forth in SEQ ID NO: 3.

(c) a nucleotide sequence set forth in SEQ ID NO: 22;

(d) the nucleotide sequence set forth in SEQ ID NO: 1;

(e) a nucleotide sequence encoding a multifunctional protein-2, wherein the hydratase activity of said multifunctional protein has been eliminated;

(f) the nucleotide sequence set forth in SEQ ID NO: 6; and

(g) a nucleotide sequence set forth in SEQ ID NO: 23.

27. The plant of claim 25 further comprising in its genome a stably integrated fourth DNA construct comprising a promoter that drives expression in a plant cell operably linked to fourth coding sequence, wherein said fourth coding sequence encodes an acetyl-CoA:acetyl transferase and is operably linked to a nucleotide sequence encoding a peroxisome-targeting signal.

28. The plant of claim 27, wherein said fourth coding sequence comprises at least a portion of the nucleotide sequence set forth in SEQ ID NO: 24.

29. The plant of claim 27 further comprising in its genome a fifth DNA construct comprising a promoter that drives expression in a plant cell operably linked to a fifth coding sequence, wherein said fifth coding sequence encodes an NADH kinase or an NAD^+ kinase and said fifth coding sequence is operably linked to a nucleotide sequence encoding a peroxisome-targeting signal

30. The plant of claim 29, wherein said fifth coding sequence comprises at least a portion of a nucleotide sequence selected from the group consisting SEQ ID NOs: 25-27.

31. The plant of claim 21, wherein said plant is an oilseed plant.

32. The plant of claim 31, wherein said oilseed plant has been genetically manipulated for the enhanced production of short-chain or modified fatty acids.

33. The plant of claim 31, wherein said oilseed plant comprises in its genome a DNA construct comprising a coding sequence for an acyl-CoA oxidase operably linked to a promoter that drives expression in a plant.

34. The plant of claim 21, wherein said plant produces polyhydroxyalkanoate in the cytosol or plastids of seeds of said plant.

35. The plant of claim 21, wherein said plant is a monocot or dicot.

36. The plant of claim 21, wherein said plant is selected from the group consisting of corn, soybean, wheat, rice, alfalfa, barley, millet, sunflower, sorghum, safflower, *Brassica sp.* and cotton.

37. Transformed seed of the plant of any one of claims 21 to 36.

38. A plant genetically manipulated to produce polyhydroxyalkanoate in its peroxisomes, said plant comprising in its genome:

a stably integrated first DNA construct comprising a promoter that drives expression in a plant cell operably linked to a first coding sequence, wherein said first coding sequence encodes a polyhydroxyalkanoate synthase and is operably linked to a nucleotide sequence encoding a peroxisome-targeting signal;

a stably integrated second DNA construct comprising a promoter that drives expression in a plant cell operably linked to a second coding sequence, wherein said second coding sequence encodes an acetyl-CoA:acetyl transferase and is operably linked to a nucleotide sequence encoding a peroxisome-targeting signal; and

a stably integrated third DNA construct comprising a promoter that drives expression in a plant cell operably linked to a third coding sequence, wherein said third coding sequence is operably linked to a nucleotide sequence encoding a peroxisome-targeting signal and said third coding sequence is selected from the group consisting of:

(a) a nucleotide sequence encoding a 3-ketoacyl-CoA reductase that is capable of utilizing NADH;

(b) a nucleotide sequence set forth in SEQ ID NO: 3;

(c) a nucleotide sequence set forth in SEQ ID NO: 22;

(d) the nucleotide sequence set forth in SEQ ID NO: 1;

(e) a nucleotide sequence encoding a multifunctional protein-2, wherein the hydratase activity of said multifunctional protein has been eliminated; and

(f) the nucleotide sequence set forth in SEQ ID NO: 6.

39. The plant of claim 38, wherein said promoters are selected from the group consisting of seed-preferred promoters, chemical-regulatable promoters, germination-preferred promoters, and leaf-preferred promoters.

40. The plant of claim 38, wherein said polyhydroxyalkanoate synthase is capable of catalyzing the synthesis of polyhydroxyalkanoate copolymers.

41. The plant of claim 38, wherein said second coding sequence comprises at least a portion of the nucleotide sequence set forth in SEQ ID NO: 24.

42. Transformed seed of the plant of any one of claims 38 to 41.

43. A method for producing polyhydroxyalkanoate comprising:

(a) growing a plant of any one of claims 21-36 and 38-41 under conditions which are favorable for the synthesis of said polyhydroxyalkanoate;

(b) harvesting said plant or parts thereof; and

(c) isolating said polyhydroxyalkanoate from said plant or said parts.

44. A plant genetically manipulated for the synthesis in its peroxisomes of at least one intermediate molecule in polyhydroxyalkanoate synthesis, said plant comprising in its genome at least one stably incorporated DNA construct comprising a

coding sequence for an enzyme involved in the synthesis of said intermediate molecule, said coding sequence operably linked to a promoter that drives expression in a plant and to a nucleotide sequence encoding a peroxisome-targeting signal, wherein said coding sequence is selected from the group consisting of:

(a) a nucleotide sequence encoding a 2-enoyl-CoA hydratase that is capable of catalyzing the synthesis of R-(-)-3-hydroxyacyl-CoA ;

(b) a nucleotide sequence set forth in SEQ ID NO: 21;

(c) a nucleotide sequence comprising the 2-enoyl-CoA hydratase domain of a multifunctional protein-2.

(d) a nucleotide sequence set forth in SEQ ID NO: 4;

(e) a nucleotide sequence set forth in SEQ ID NO: 1,

(f) a nucleotide sequence encoding a multifunctional protein-2, wherein the dehydrogenase activity of said multifunctional protein has been eliminated; and

(g) a nucleotide sequence encoding a 3-ketoacyl-CoA reductase that is capable of utilizing NADH;

(h) a nucleotide sequence set forth in SEQ ID NO: 3;

(i) a nucleotide sequence set forth in SEQ ID NO: 22;

(j) the nucleotide sequence set forth in SEQ ID NO: 1;

(k) a nucleotide sequence encoding a multifunctional protein-2, wherein the hydratase activity of said multifunctional protein has been eliminated; and

(l) the nucleotide sequence set forth in SEQ ID NO: 6.

45. The plant of claim 44, wherein said intermediate molecule is an R-(-)-3-hydroxyacyl-CoA or a 3-ketoacyl-CoA.

46. Transformed seed of the plant of claim 44 or 45.

47. A transformed plant cell comprising in its genome at least one stably integrated DNA construct comprising a nucleotide sequence operably linked to a

promoter that drives expression in a plant cell, wherein said nucleotide sequence is selected from the group consisting of:

(a) the nucleotide sequence set forth in SEQ ID NO: 1;

(b) a nucleotide sequence encoding the amino acid sequence set forth in SEQ ID NO: 2;

(c) a nucleotide sequence having at least 50% sequence identity to the nucleotide sequence set forth in SEQ ID NO: 1;

(d) an antisense nucleotide sequence corresponding to the nucleotide sequence of (a), (b) or (c); and

(e) a nucleotide sequence that hybridizes under stringent conditions to the nucleotide sequence of (a).

48. A plant cell genetically manipulated to produce polyhydroxyalkanoate in its peroxisomes, said plant cell comprising in its genome:

a stably integrated first DNA construct comprising a promoter that drives expression in a plant cell operably linked to a first coding sequence, wherein said first coding sequence encodes a polyhydroxyalkanoate synthase and is operably linked to a nucleotide sequence encoding a peroxisome-targeting signal; and

a stably integrated second DNA construct comprising a promoter that drives expression in a plant cell operably linked to a second coding sequence, wherein said second coding sequence is operably linked to a nucleotide sequence encoding a peroxisome-targeting signal and said second coding sequence is selected from the group consisting of:

(a) a nucleotide sequence encoding a 2-enoyl-CoA hydratase that is capable of catalyzing the synthesis of R-(-)-3-hydroxyacyl-CoA ;

(b) a nucleotide sequence set forth in SEQ ID NO: 21;

(c) a nucleotide sequence comprising the 2-enoyl-CoA hydratase domain of a multifunctional protein-2.

(d) a nucleotide sequence set forth in SEQ ID NO: 4;

(e) a nucleotide sequence set forth in SEQ ID NO: 1; and

(f) a nucleotide sequence encoding a multifunctional protein-2, wherein the dehydrogenase activity of said multifunctional protein has been eliminated.

49. The plant cell of claim 48, wherein said polyhydroxyalkanoate synthase is encoded by a nucleotide sequence selected from the group consisting of SEQ ID NOs: 8-12.

50. The plant cell of claim 48 further comprising in its genome a stably integrated third DNA construct comprising a promoter that drives expression in a plant cell operably linked to a third coding sequence, wherein said third coding sequence encodes a 3-ketoacyl-CoA reductase and is operably linked to a nucleotide sequence encoding a peroxisome-targeting signal.

51. The plant cell of claim 50, wherein said third coding sequence comprises at least a portion of a nucleotide sequence selected from the group consisting of:

(a) a nucleotide sequence encoding a 3-ketoacyl-CoA reductase that is capable of utilizing NADH;
(b) a nucleotide sequence set forth in SEQ ID NO: 3;
(c) a nucleotide sequence set forth in SEQ ID NO: 22;
(d) the nucleotide sequence set forth in SEQ ID NO: 1;
(e) a nucleotide sequence encoding a multifunctional protein-2, wherein the hydratase activity of said multifunctional protein has been eliminated;
(f) the nucleotide sequence set forth in SEQ ID NO: 6; and
(g) a nucleotide sequence set forth in SEQ ID NO: 23.

52. The plant cell of claim 50 further comprising in its genome a stably integrated fourth DNA construct comprising a promoter that drives expression in a plant cell operably linked to fourth coding sequence, wherein said fourth coding sequence encodes an acetyl-CoA:acetyl transferase and is operably linked to a nucleotide sequence encoding a peroxisome-targeting signal.